Myoactive neuropeptides in Manduca sexta

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MYOACTIVE NEUROPEPTIDES IN *MANDUCA SEXTA*

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I dedicate this work to my parents who have given me so much support, encouragement and love over the years and have faced adversity with such courage and fortitude.

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Abstract

The regulatory mechanisms governing the spontaneous contractions of visceral muscles in insects are not well understood. Most viscera contract rhythmically but the rate of contraction must be altered according to requirements imposed by environmental conditions or the physiological status of the insect. Nervous or neurohormonal input is implicated.

In this study, three *in vitro* bioassay systems from *Manduca sexta* are used to investigate the effects of a range of neuropeptides, neurotransmitters and endogenous myoactive factors on visceral muscles of this insect. The screening of a range of neuropeptides using adult and larval heart bioassays show that a number of peptides are cardioactive including; crustacean cardioactive peptide, allatotropin, locustatachykinin II, members of the FLRFamide family, corazonin, leucopyrokinin and two of the callatostatins. This suggests that the heart has receptors to structurally similar peptides and that identical or structurally similar peptides are present in *Manduca sexta*. Some peptides were found to be selectively active in either the adult heart (allatotropin) or larval heart (locustatachykinin II, leucomyosuppressin, pheromone biosynthesis activating neuropeptide and leucopyrokinin) therefore cardiac regulation is dissimilar in the two developmental stages.

A novel bioassay employing the oviduct of *Manduca sexta* is described. From a range of neuropeptides and neurotransmitters; crustacean cardioactive peptide, allatotropin, dopamine, gamma amino butyric acid and serotonin were found to be myoactive in the oviduct. No inhibitory substances were found.

A screening of HPLC fractions from the separation of both *Manduca sexta* and *Schistocerca gregaria* nervous tissue extracts reveals a number of myoactive fractions. The responses to these fractions of the adult and larval heart and the oviduct preparations and the elution times of synthetic peptides under identical separation conditions enables some of the active substances to be identified. The peptide CCAP was detected in *Manduca sexta* nervous system extracts, eluting at the same time as synthetic CCAP and active in all three bioassays. Masallatotropin was detected in *Manduca sexta* whole head extracts eluting at the same time as the synthetic peptide but myoactive only in the adult heart and oviduct bioassays. A number of other myoactive factors were present in extracts from both insect species, of particular note is a previously unreported cardiosuppressive neuropeptide from *Manduca sexta* brains. Further identification of the peptides involved was beyond the scope of this study.

The pattern of innervation of the oviduct is examined in some detail. Immunohistochemical investigation and HPLC separation of extracts of the nerves which extend to the female reproductive system followed by testing of the subsequent HPLC fractions, implicates CCAP as an important neuropeptide involved in the regulation of oviduct motility *in vivo*.

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Chapter 1

Introduction – The Visceral Muscles of Insects

1.1 Introduction

The term visceral is extended to all those muscles which invest the internal organs as opposed to the somatic or skeletal musculature. They are not under the direct control of the animal but rather are involuntary. Many visceral muscles contract rhythmically and spontaneously, and this activity is governed by myogenic centres within the muscle fibres or by input from the sympathetic nervous system. Unlike vertebrate visceral muscles which form a distinct structural group in that most lack striations and are smooth, insect visceral muscle is striated in a similar way to somatic muscles. Insect muscle is therefore functionally rather than structurally divided into visceral and somatic types.

The definition of insect visceral muscles used here will include those of the circulatory system such as the heart, aorta, segmental vessels, secondary pulsatile organs and alary muscles of both the pericardial septum and ventral diaphragm, those of the alimentary canal including the Malpighian tubules and those of the reproductive system. Visceral muscles of the various glands, the spiracular mus-

cles and other muscles involved in respiration will not be included here.

Most insect visceral muscles undergo myogenic contractions. Stretching, nervous stimulus and neurohormonal input can all cause alterations in the rhythmic, spontaneous contraction rate. The ability of visceral muscle to undergo spontaneous rhythmic contractions following isolation from the insect, and to respond to exogenous myoactive factors has resulted in the extensive use of various insect visceral muscles in bioassay systems for the detection of neurotransmitters and neuropeptides.

In recent years it has become apparent that myotropic neuropeptides play a considerably more important role in physiological processes than was first thought. Cameron (1953) discovered that substances present in the neuroendocrine system of *Periplaneta americana* stimulated visceral muscle. This discovery led to attempts to isolate and characterise factors causing contractile activity. Davey (1962) described the effects of homogenates of the corpus cardiacum on the contractile activity of the hindgut and Brown (1965) showed that three peptides were responsible for most of the observed myotropic activity. P1 was active in both the heart and hindgut and P2 and P3 were specific stimulators of the hindgut and heart respectively. In further studies, Brown (1967) detected another stimulatory substance in extracts of hindgut with high specific activity in extracts of the proctodeal nerve. Proctolin was the first insect neuropeptide to be isolated (Brown 1975, 1977) and sequenced (Starratt and Brown 1975).

The development of HPLC has made possible the rapid and efficient isolation of peptides from tissue extracts. The number of peptides isolated from insects and known to be active on insect visceral muscle is now considerable, however rather less is known about the physiological mechanisms of neuromodulatory control by

these peptides. From the response of isolated target tissues in bioassay systems it is not possible to determine the activity of a substance *in vivo*. One tissue may respond in a similar way to a large range of neuropeptides (for example the *Leucophaea maderae* hindgut (review: Holman *et al.* 1991)) or a single peptide may stimulate a number of different visceral muscles in the same insect for example crustacean cardioactive peptide (CCAP) in the locust hindgut (Stangier *et al.* 1989), oviduct (Burdzik 1990) and heart (Dircksen *et al.* 1991). The identified insect neuropeptides have a tendency to occur in structural families and different family members may have different characteristic bioactivities when tested in the same tissue. For example the activity of the FLRFamide-like peptide family from *Locusta migratoria* (Peeff *et al.* 1993, Lange *et al.* 1994) in the oviduct of this insect. Some of these peptides are myostimulatory, others inhibit sponaneous contractions and others cause biphasic responses with both stimulatory and inhibitory activity. Some neuropeptides which were initially isolated by virtue of their myotropic activity, have since been shown to have other physiological functions such as in water balance, pheromone production, melanization, diapause and juvenile hormone biosynthesis.

The question as to why so many peptides are required for the regulation of seemingly simple processes is, as yet, unanswered. Perhaps these processes are not as simple as they might at first appear. The bioassay is a crude tool in the investigation of physiological responses and differences in the responses to myoactive factors are likely to be at the cellular rather than whole tissue level. The study of the activity and the localisation of myotropic factors in the nervous system does not, as yet, explain the regulation of muscle contraction in insects. Nevertheless, this work provides a valuable basis from which to conduct more detailed studies of the cellular mechanisms involved and then to relate these to the effects observed *in vivo*.

In this introductory chapter a general overview of the structure, function and endogenous regulation of visceral muscle in insects is presented. The heart and reproductive visceral muscles of *Manduca sexta*, the species with which much of the remainder of the thesis is concerned, are examined in greater detail and a summary of the experimental work covered in the following chapters is included.

1.2 The Structure and Function of Insect Visceral Muscle

1.2.1 The Circulatory System

The Heart

Haemolymph bearing nutrients, ions and metabolic waste is circulated around the insect by a system of pumps of which the most important is the dorsal vessel.

The dorsal vessel consists of a tube which lies along the length of the dorsal midline of the abdomen and may extend into the thorax. The 'heart' is usually taken to be the abdominal portion, which is the more contractile part and also has pairs of valved openings or ostia which permit the uptake of haemolymph. The heart has the appearance of being segmentally chambered, with the posterior of each chamber enlarged dorsally to form an ampulla. Each segmental division has a pair of ostia. The number of chambers varies greatly between species, for example, in *Periplaneta* the twelve heart chambers consist of nine in the abdomen and three in the posterior of the thorax, *Schistocerca* has eight chambers and in some Odonata the heart is reduced to a single chamber with one pair of ostia (review: Jones 1977).

In most insects the heart is a simple tube structure with no internal valves to prevent backflow. Haemolymph enters the ostia during the relaxation phase or diastole then these valves prevent haemolymph from leaving during contraction or systole forcing it forward to the anterior end of the aorta and then into the haemocoel. The resultant increase in pressure at the anterior leads to the backwards movement of haemolymph in the body cavity.

The activity of the heart is myogenic. Isolated hearts and even fragments of the heart will continue to contract rythmically and the beat is propagated by the cardiac muscles.

The wave of contraction usually starts at the posterior end of the heart. Haemolymph is pumped forward by the heart into the thorax and exits via the aorta into the head. The appendages of the head and thorax are supplied by the circulating blood through systems of tubes, septa, valves and pumps. Accessory pulsatile organs, with similar characteristics to the heart are present in appendages such as the legs and at the base of the antennae (review: Jones 1977). In Lepidoptera and Diptera reversal of the heartbeat commonly occurs in larvae and pupae (review: Jones 1977) with the anterograde contractions typically being faster than the reterograde. The reason for this phenomenon is not clear, however Wasserthal (1975) found a characteristic pattern of heartbeat reversals just prior to and during adult eclosion and during wing expansion in *Caligo brasiliensis*, suggesting that reversals were of importance for circulation at these stages of development.

The Alary Muscles and the Dorsal Diaphragm

The heart chambers are attached to the integument by radiating filaments of connective tissue and pairs of transversely oriented alary muscles which, together with the dorsal diaphragm, form the pericardial septum. The muscles are arranged in a fan-like pattern being widely spread under the heart and converging at the body wall. Haemolymph enters the pericardial sinus in which the heart lies, through openings in the diaphragm and/or at the posterior end of the diaphragm, then moves into the dorsal vessel through the ostia during the relaxation phase of the heart. The contractions of the alary muscles do not affect the heartbeat and no functional interrelationship has been shown between the two.

The Ventral Diaphragm

In many insect orders and particularly in Lepidopterans a fibro-muscular septum stretches across the ventral nerve cord enclosing a perineural sinus. The membrane undergoes peristaltic contractions in such a way as to cause the blood below to flow in a posterior and lateral direction. The ventral diaphragm is of particular importance in insects that use the circulation in thermoregulation but also allows the rapid exchange of humoral factors between the nerve cord and the haemolymph both by aiding in the flow of haemolymph and by agitation of the nerve cord.

1.2.2 The Digestive System

The Foregut

The insect foregut typically consists of the pharanyx, the oesophagus, the crop and the proventriculus, with the regions being variously developed depending on the feeding habits of the species. The function of the foregut is the ingestion, storage, grinding and transport of food to the midgut. The foregut is made up of a cuticular lining which may be smooth or may bear hairs, spines or teeth. The cuticular lining is secreted by a thin epidermis on a basement membrane. Surrounding this is a thick muscular coat with longitudinal muscles inside and circular outside and this is enclosed by a connective tissue sheath containing nerves and tracheae. In some insects longitudinal muscles are also present inside the layer between the folds of the epithelium

The contractile activity of the foregut is complex. In *Galleria* larvae (Beard 1960) the proventriculus was shown to have both a constant pulsating activity and an intermittent, independent contraction pulse from posterior to anterior. Peristaltic waves ran from the crop/proventriculus junction forwards to the oesophagus and more strongly backwards. Independent peristaltic activity occured in the oesophagous. The activity of the foregut is constant (Oldfield and Huddart 1982) and it has been observed that spontaneous contractions continue after prolonged starvation of locusts and cockroaches.

The Midgut

The midgut is the site where most digestion occurs. The epithelial cells produce and secrete digestive enzymes and absorb the resultant breakdown products. It consists of a tube which may bear four, six or eight caecae at the anterior end. The midgut is not lined by cuticle but, in many insects, the epithelium is protected from the contents of the gut by a thin sheath, the peritrophic membrane. This consists of a network of chitin fibrils in a protein-carbohydrate matrix and is produced either from the epithelial cells along the length of the midgut or by cells at the anterior of the midgut. The function of the membrane is to act as a sieve for food particles, bacteria and large molecules. Digestive enzymes and the products of digestion pass through readily to the midgut epithelial cells.

The musculature of the midgut is varied. In the mosquito (Schaefer *et al.* 1967) and the blowfly, *Calliphora erythrocephala* (Saleh 1979) a single layer of muscle cells forms a random network. In *Sarcophaga* (Nopanitaya and Misch 1974), two layers of muscles occur with an inner circular layer and an outer longitudinal. In *Schistocerca* (Anderson and Cochrane 1977, 1978), layers of internal and external longitudinal muscles surround the layer of circular muscles. The action of the midgut muscles is a churning and peristaltic movement.

The Hindgut

The start of the hindgut is marked by the entry of the Malpighian tubules into the alimentary canal, often at the pylorus. The hingut is made up of the ileum, colon and rectum and its function is the absorption of water, salts and other substances from the faeces and urine. The structure is similar to that of the

foregut, however the cuticular layer is thinner and is permeable to water.

The ileum and colon are less muscular than the rectum usually with inner circular and outer longitudinal layers. Along the rectum the longitudinal muscles are often collected into strands opposite the rectal pads. The rectum undergoes complex movements of compression, peristalsis and reverse peristalsis (Cook and Reinecke 1973) brought about by network of both circular and longitudinal muscles. Movement is also influenced by extrinsic visceral muscles, the rectal dilator muscles.

The Malpighian Tubules

The Malpighian tubules are long blind-ending tubes, attached at the open end to the junction of the mid- and hindgut and with the distal end lying free in the haemolymph. They function as the main organs of excretion and osmoregulation in insects. The tubule wall is only one cell thick on a basement membrane to which muscles are attached in some orders. In the Thysanura, Dermaptera and Thysanoptera no muscles are present. In the Lepidoptera, Diptera, Trichoptera and some Hemiptera muscles at the base whip the tubules around in the haemolymph whereas in the Coleoptera and Neuroptera a muscle coat causes peristaltic movements. In the Orthoptera and Odonata spirals of muscle fibres around the tubules contract resulting in writhing movements and helical coiling (Palm 1946).

The Malpighian tubules are not innervated and the muscles exhibit rhythmic contractions even after complete isolation and are therefore myogenic. Any other controls over movement must be humorally derived.

1.2.3 The Reproductive System

The visceral muscles of the reproductive system are the means by which the sperm are stored, then transported from the male to the female and then stored once again before fertilisation takes place. Visceral muscles move the eggs from the ovarioles into the lateral oviducts then to the common oviduct and also play a major role in oviposition.

The Female System

Figure 1.1 shows a diagram of a generalised insect female reproductive tract. In the female insect the paired ovaries are made up of a number of ovarioles which consist of chains of developing ova sheathed in a thin structureless basement membrane, the tunica propria. In some insects there is a layer of flat cells on the outside of the tunica, the epithelial sheath. A thin layer of longitudinal muscles may extend up the walls of the ovarioles from the lateral oviducts. The ovarioles open into the lateral oviducts. These are tubes with a single layer of cuboid or columnar cells on a basement membrane with a muscle layer outside. In the stick insect, *Carausius* (Thomas 1979), the lateral oviducts consist of a sheath of epithelial cells on a basement membrane surrounded by internal and external layers of longitudinal muscle and a central circular muscle layer, but in the horsefly *Tabanus sulficrons* (Cook and Meola 1978) a single layer of longitudinal muscles is present.

At the anterior end of each lateral oviduct where the ovarioles enter is often an expanded area, the calyx. Generally, the two lateral oviducts join at a muscular common oviduct which originates from the ectoderm and has a cuticular lining.

The muscles of the common oviduct of *Tabanus sulficrons* (Cook and Meola 1978), *Zygogramma exclamationis* (Gerber *et al.* 1978) and *Ephestia kuhniella* (Cruickshank 1973) are in two layers with an inner network of longitudinal fibres and an outer of circular. In *Carausius* (Thomas 1979) a single layer of semicircular muscle, not completely sheathing the oviduct, is present. The common oviduct extends posteriorly to the vagina and its opening, the vulva. The bursa copulatrix is a pouch sheathed in a thin layer of circular muscles and enclosing a thick chitinous interior. It opens off the vagina and receives the penis during copulation but is not present in many insects. The spermatheca is also a pouch which functions to store the sperm from the time of mating until required for fertilisation of the eggs. Studies of the spermatheca in *Periplaneta americana* (Gupta and Smith 1969) show that this gland possesses two to three layers of small visceral muscle fibres mainly longitudinally orientated and a large number of nerve endings are present on the gland with both myoneural and neurosecretory endings.

Accessory glands are usually present, the contents of which may be used by different species to produce a variety of substances such as adhesive to allow the attachment of the eggs to the substratum, tanning proteins and frothy or gelatinous egg sheaths. The fine structure of these glands has not been well studied.

The Male System

The main functions of the male reproductive system are the production and storage of sperm and their transport to the female reproductive tract in a viable condition. Figure 1.1 shows a diagram of a generalised insect male reproductive tract. Generally the male has paired testes linked by tubular sperm ducts or vasa

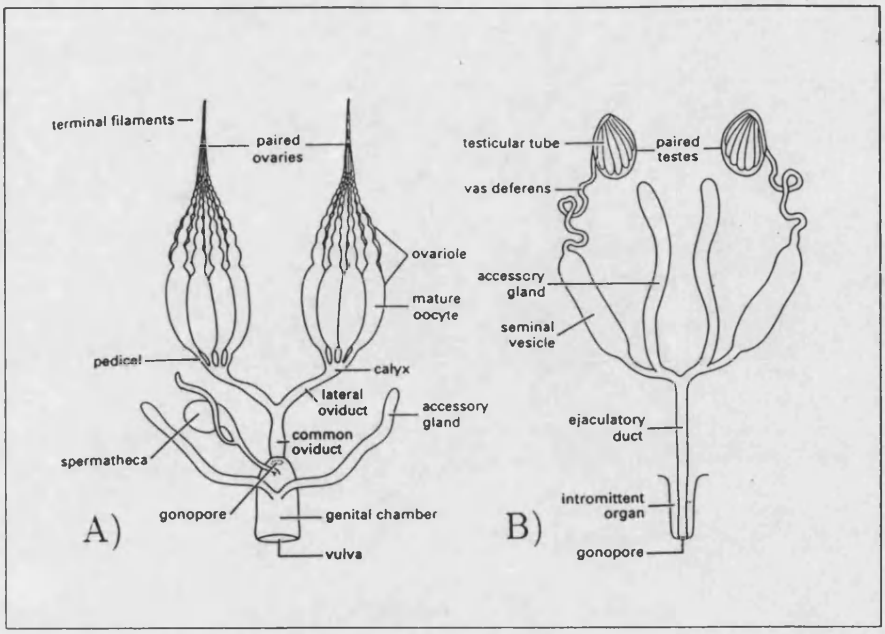

Figure 1.1: Generalised insect reproductive tracts.
A) female and B) male (adapted from Snodgrass 1935).

deferentia to the seminal vesicles or sperm storage organs. Each testis typically consists of a number of short germ tubes or testicular follicles containing male germ cells in successive stages of development. Paired accessory glands may be present on the vasa deferentia or in some insects, the tubules themselves are glandular or in others, the accessory glands open off the ejaculatory duct. The accessory glands are usually elongate sacs or tubes and the tubular forms are extensively looped and coiled. The paired vasa deferentia converge into the ejaculatory duct which carries the sperm either as semen or as a sperm package to the intromittent organ, the penis, then to the gonopore and finally to the female.

In general, most parts of the male reproductive tract are sheathed in inner circular and outer longitudinal muscle sheets. In the sunflower beetle, *Zygogramma exclamationis*, Gerber *et al.* (1978) describe the median ejaculatory duct as possessing a thick layer and each sperm duct as having a thin layer of circular muscle. A thin circular muscle sheet covers the accessory glands and the vasa deferentia

but the testes are sheathed in an epithelial layer with no muscular component.

The products of the male accessory glands are involved in the formation of the spermatophore, contribute to the seminal fluid which nourishes the sperm in transit, induce activation (sperm motility) and also are likely to influence the behaviour of the female.

1.3 Modulation of Visceral Muscle Motility

1.3.1 The Circulatory System

The Heart and Alary Muscles

That the insect heart beats myogenically, such that the contractions are governed by pacemakers within the muscle fibres rather than neurogenically, by nervous input from central or peripheral pacemakers, has been shown in many species (McCann 1970). The heartrate does not remain constant however, and varies according to age, sex, nutritional status, stress and environmental conditions (review: Jones 1977). A system which acts to modulate the myogenic rhythm generated in the myofibrils is therefore implicated.

Innervation of the dorsal vessel in insects appears to be variable. In the cockroach two sets of nerves are involved in cardioregulation, paired lateral cardiac nerve cords and segmental nerves from the ventral nerve cord. Both of these nerve types contain conventional electron-lucent vesicles (Johnson 1966, Miller 1968) and neurosecretory nerve cells. A similar pattern of innervation by lateral car-

eral cardiac nerves and segmental nerves is described for *Bombyx mori* (Kuwana 1932, McIndoo 1945) but in the adult moth of *Hyalophora cercropia* no evidence of cardiac innervation was noted (Sanger and McCann 1968a, b). The hearts of the moths *Sphinx ligustri* and *Ephestia kuehniella* and the butterfly *Caligo beltrao* are multiterminally innervated by branches from the transverse segmental nerves (Wasserthal and Wasserthal 1977, 1980) with no lateral cardiac nerves being present in Lepidopterans. All the branches contain single axons with electron-lucent vesicles but the thickest branches also contain electron dense neurosecretory axons. Along the segmental nerve branches and in the heart, close to the ostia, are specialised regions morphologically consistent with neurohaemal release sites, strongly suggesting that a neurosecretory nervous network is involved in regulation of the heart.

The alary muscles are innervated via the segmental nerves in the cockroach (Adams *et al.* 1973, Miller and Thomson 1968). Stimulation of these nerves induced muscle contraction. The axons contain both electron-dense and electron-lucent vesicles. In the locust, the alary muscles are neurally driven rather than myogenic (Miller *et al.* 1979, Miller and Rozsa 1981). The alary muscles of *Sphinx lingustri* are multiterminally innervated by branches of the transverse segmental nerves and only electron-lucent vesicles are present in the axons which make junctions on the muscle fibres (Wasserthal and Wasserthal 1977). In *Sphinx* innervation of the alary muscles is much greater than that of the heart cells suggesting that these muscles have an important role in cardiac regulation.

Modulation of the Heart in *Manduca sexta*.

Taghert (1981) described the anatomy of the abdominal segmental nerves in *Manduca sexta* (see Fig. 1.2). Each ganglion has paired segmental nerves, the dorsal (DN) and ventral nerves (VN). Transverse nerves (TN) arise from the unpaired median nerve which runs between the two connectives from each ganglion to the next and anastomose with the VN of the preceding ganglion. The perivisceral organs are slight swellings of the transverse nerve which then innervates the closer muscle of the spiracle. The TN anastomoses with the DN of the next ganglion then projects to the heart and alary muscles.

Davies *et al.* (1993), noted the presence of peripheral neurosecretory cells on the distal TN of each abdominal segment.

Functional innervation of the heart of *Manduca sexta* has not been shown (S. E. Reynolds, N. J. Tublitz, personal communication) suggesting that regulation of the heartbeat is achieved by circulating neurohormones released from neurohaemal sites. The central nervous system of this moth is known to contain a group of myoregulatory peptides, the CAPs (cardioacceleratory peptides) which have been shown to elevate the heartrate during wing inflation and flight (Tublitz and Evans 1986, Tublitz 1989). The CAPs consist of two groups of peptides, the CAP1s and the CAP2s. One of the CAP2s has been isolated and sequenced (Cheung *et al.* 1992) and is known to be identical to crustacean cardioactive peptide (CCAP) (Stangier *et al.* 1987).

An immunological study of the distribution of the cardioactive peptide, CCAP in *Manduca sexta*, has revealed that abdominal neurosecretory cells project posteriorly to neurohaemal release organs (Davies *et al.* 1993). A schematic rep-

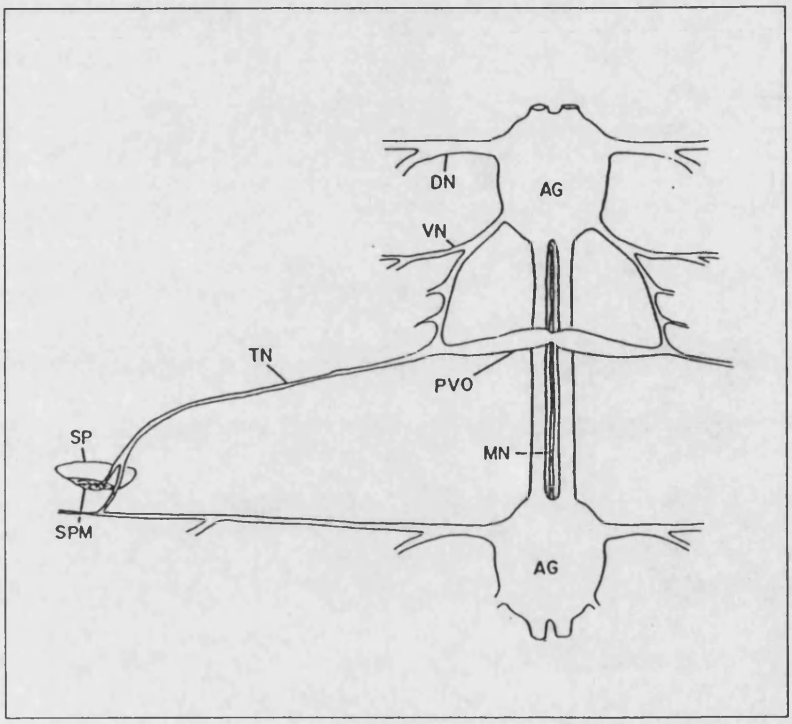

Figure 1.2: Gross anatomy of the transverse nerve in adult *Manduca sexta.* *The segmental abdominal ganglia (AG) have two segmental nerves, the dorsal nerves (DN) and the ventral nerves (VN). The transverse nerve (TN) comes from the unpaired median nerve (MN) which runs between the two connectives, the proximal end of the TN is the perivisceral organ (PVO). The TN links via anastomosis with both the VN of the preceding ganglia and the DN of the following ganglion and innervates the spiracle closer muscle (SPM) before proceeding to the alary muscles of the heart. From Taghert 1981.*

resentation of the pathway of the CCAP immunoreactive neurosecretory cells in the abdomen of *Manduca sexta* is shown in Fig. 1.3.

The axon of a CCAP containing cell extends to the next ganglion and enters the dorsal nerve (DN). The DN branches and the ventral anterior branch joins with the transverse nerve (TN). The CCAP axon bifurcates at the junction with one branch extending along the TN to the perivisceral organ (PVO), which is known to be a major site of neurosecretion in the abdomen of insects (Raabe 1982). The other branch of the axon projects past the spiracle to a peripheral neurosecretory cell from which axons project both to the alary muscles of the heart and to the PVO. According to Davies *et al.* (1993), the heart is not innervated by CCAP immunopositive axons.

The evidence suggests that in *Manduca sexta* CCAP acts as a circulating neurohormone in the regulation of the heartrate and has a neurotransmitter role on the alary muscles.

The Ventral Diaphragm

Study of the activity of the ventral diaphragm has been limited because of the difficulty of separating the muscle from the nervous system without damage in many insect species.

In the cockroach *Periplaneta*, innervation of the hyperneural muscle, the vestigial ventral diaphragm has been examined in detail (Miller and Adams 1974). The muscle is innervated by transverse segmental branches of the ventral median nerve of the ventral nerve cord. Both conventional electron-lucent and electron-dense

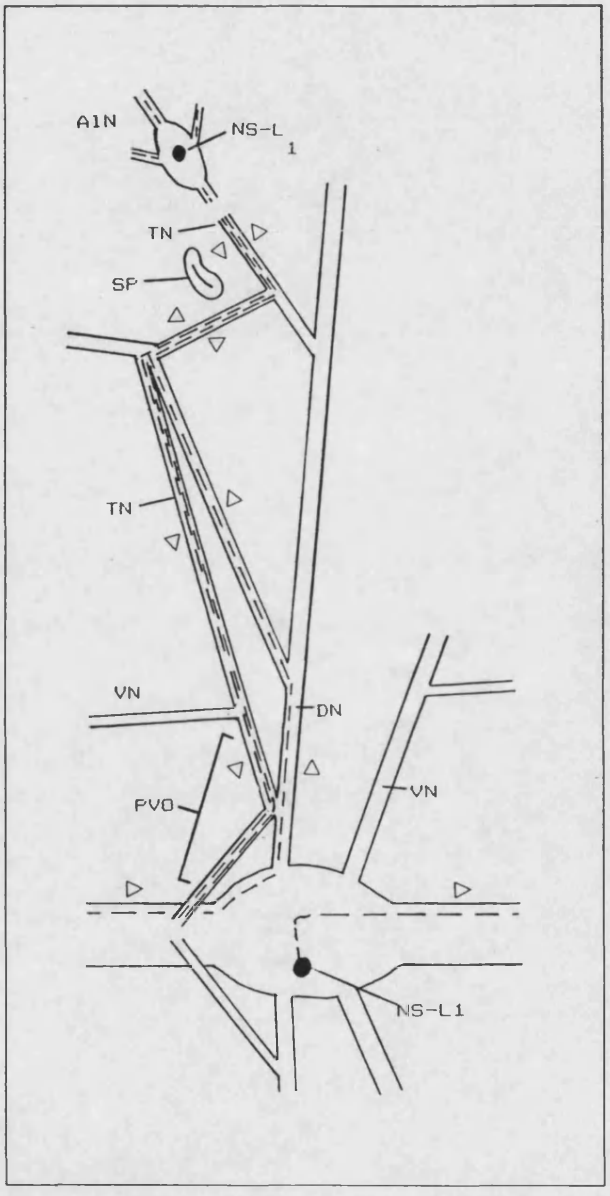

Figure 1.3: Schematic representation of the pathway of the CCAP immunoreactive neurosecretory cells in the abdominal nerve cord of *Manduca sexta*.
AlN – nerves innervating alary muscles, NS-L1 – peripheral neurosecretory cell (enlarged), TN – transverse nerve, SP – spiracle, DN – dorsal nerve, PvO – perivisceral organ, VN – ventral nerve and NS- L_1 – CCAP immunopositive neurosecretory cell of the abdominal ganglia (not to scale) (adapted from Davies et al. 1993).

vesicles are present although no evidence for the release of the electron-dense material by exocytosis was noted. The hyperneural muscle only shows spontaneous activity when the nerve cord is left attached and contractions mediated by proctolin require the presence of the nerve cord (Miller 1979) suggesting that contractions of the cockroach hyperneural muscle are neurogenic.

In the locust each segment of the ventral diaphragm is innervated by four motor neurones (Peters 1977). Two of these are derived from the corresponding abdominal ganglia for each segment and the other two are branches from the nerve in the preceding segment. As in the cockroach, the muscle fibres are not spontaneously active without innervation. Immunohistochemical studies of the innervation of the ventral diaphragm in this insect has revealed that CCAP immunopositive axons have endings on these muscles (Dircksen *et al.* 1991).

1.3.2 The Digestive System

Innervation of the alimentary canal of insects is not consistent but generally follows the pattern that the fore and midguts are innervated by the stomatogastric system and the hindgut by the caudal sympathetic system, that is nerves arising from the composite terminal ganglia. The stomatogastric system is made up from various small ganglia and associated nerves; the frontal ganglion linked to the tritocerebrum, a medial hypocerebral ganglion, paired or unpaired ventricular (ingluvial) ganglia and in some insects paired proventricular ganglia close to the junction of the mid and hindguts (see Fig. 1.4).

The frontal ganglion lies at the front of the brain above the oesophagus and is joined via two bilateral connectives to the tritocerebral lobes. A recurrent nerve

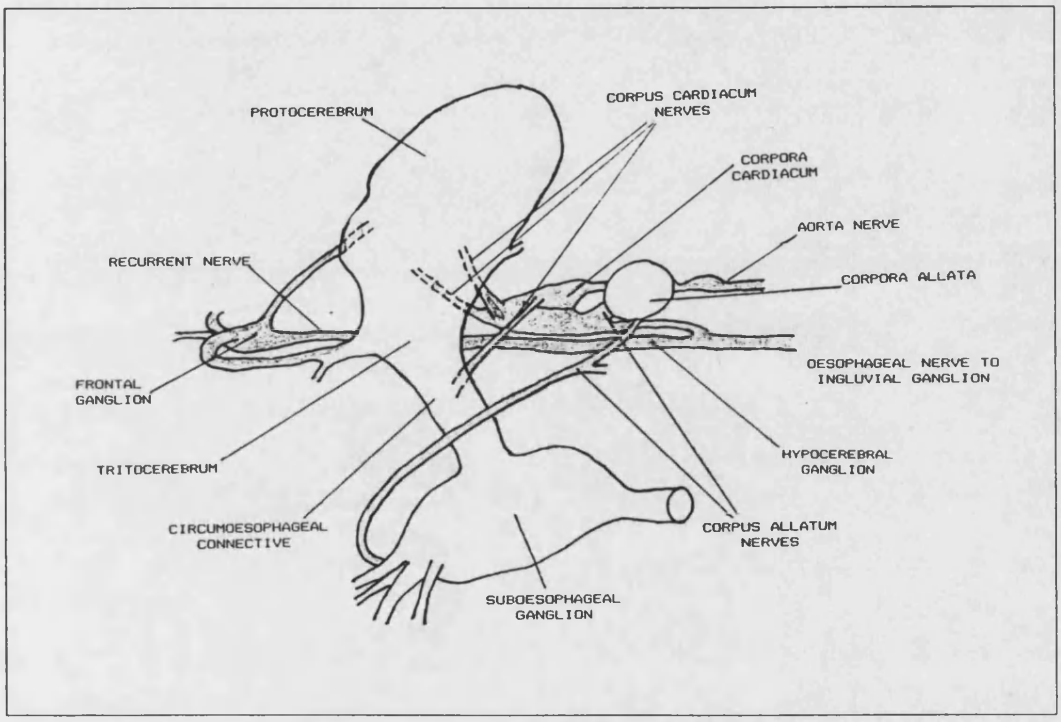

Figure 1.4: Figure showing lateral view of the brain, suboesophageal ganglion, stomatogastric nervous system and corpus allatum of the cockroach.
(after Willey 1961, from Wigglesworth 1972)

passes between the brain and the oesophagus posteriorly to the hypocerebral ganglion lying behind the brain. The hypocerebral ganglion is connected laterally to the corpora cardiaca and to the brain via the nervi corpori cardiaci. This ganglion innervates the oesophagus and is connected to the ingluvial ganglia, located on the foregut via the outer oesophageal nerve. In, for example *Periplaneta*, a pair of ingluvial nerves pass back to the proventriculus and terminate in a proventricular ganglion which innervates the entire foregut and the anterior of the midgut (Willey 1961). In other insects, for example the fire ant, *Solenopsis*, the proventriculus and foregut are innervated by the ingluvial ganglia (Petralia and Vinson 1980).

Studies have shown that the fore and midguts are likely to be controlled by neurohormones in addition to conventional neurotransmitters. In both *Blaberus* (Chanussot 1972) and *Locusta* (Anderson and Cochrane 1977, 1978) the presence of neurosecretory vesicles in the ingluvial ganglia suggest that these may play a neurohaemal role .

A number of peptides, identical to myotropic peptides previously isolated from the nervous system of the same insect, have been isolated from midgut extracts of *Locusta migratoria* (Schoofs *et al.* 1989, 1991c). In *Manduca sexta* two novel pentapeptide myotropins have been isolated from larval midguts (Yi *et al.* 1992, Yi *et al.* 1995).

In most insects studied, the hindgut receives innervation via the proctodeal nerves from the terminal abdominal ganglia (Brown and Nagai 1969, Dunbar 1980, Reinecke and Adams 1977). These ramify over the hindgut but the nerve endings do not penetrate the basement membrane of the gut epithelium and so are not considered to be sensory but rather are motor neurones. In studies of the hindgut

of the locust (Dunbar 1980), two types of axon were noted, one with membrane bound electron dense neurosecretory granules and the other with no granules.

In *Manduca sexta* larvae (Reinecke *et al.* 1978), branches of the proctodeal nerves innervate the pylorus, ileum and colon. A small plexus is present on either side of the rectal valve at the junction of the dorsal nerve branch and the proctodeal nerve. The plexus neurones contain neurosecretory granules and their axons run along the proctodeal nerve to the colon. The neuromuscular junctions on the outer circular muscle contain many neurosecretory granules which may be released directly into the muscle sarcoplasm.

The evidence suggests that the motility of the hindgut in insects is likely to be modulated both by neurosecretory products and conventional neurotransmitters (Cook and Holman 1980).

The hindgut of the Madeira cockroach, *Leucophaea maderae* has been used extensively as a screening tool for myotropic peptides both from this species and others (review: Holman *et al.* 1991) and has been found to respond to a large number of neuropeptides. From *Leucophaea maderae* itself, the leucokinins I-VIII (Holman *et al.* 1986a,b, 1987a,b), leucopyrokinin (Holman *et al.* 1986b), leucosulfakinins I-II (Nachman *et al.* 1986a,b) and leucomyosuppressin (Holman *et al.* 1986d) are myoactive in the cockroach hindgut. The achetakinins I-V from head extracts of *Acheta domesticus* were also isolated using the *Leucophaea* hindgut bioassay (Holman *et al.* 1990b). In the locust *Locusta migratoria*, the locustatachykinins I-V (Schoofs *et al.* 1990a,c), locustamyotropins I-IV (Schoofs *et al.* 1990d,e, 1992a), locustasulfakinin (Schoofs *et al.* 1990b), locustapyrokinin (Schoofs *et al.* 1991a), locustakinin (Schoofs *et al.* 1992b) and locustamyosuppressin (Schoofs *et al.* 1993a) are all active in the *Leucophaea* hindgut.

Immunological studies have shown that leucokinin-I (LK-I) immunopositive neurons do not innervate the hindgut of *Leucophaea* directly (Nassel *et al.* 1992). Anti-locustamyotropin (LM-MT) antisera stained cells in the stomatogastric system and also processes from immunopositive abdominal ganglia cell bodies which extended to the neurohaemal organs in *Locusta migratoria* suggesting firstly a role for these peptides in control of fore and midgut motility and also a neurohormonal action in the hindgut (Schoofs *et al.* 1992c).

Clearly the *Leucophaea* hindgut responds to a considerable range of known neuropeptides indicating that a number of receptor types are present in the muscles. The reason why such a large range of peptides is detected and their relationship to hindgut motility *in vivo* is not clear. Many of the peptides belong to structurally related families and so the active amino acid sequence core would be likely to bind to the same receptor. Different peptides may have slightly different actions at the cellular level or, different peptides of the same structural family may be restricted to specific locations in the insect. One conclusion which can be drawn from peptide isolation studies using the insect hindgut is that regulation of hindgut and probably other insect visceral muscle motility is likely to be more complex than first expected, requiring many control factors not just one or two.

1.3.3 The Reproductive System

The Female

The female reproductive system is innervated by the terminal ganglia of the abdominal nerve cord. Cook *et al.* (1980) describe the pattern of innervation seen in the female reproductive system of the pink bollworm *Pectinophora gossypiella*

which was nearly identical to that described in *Manduca sexta* (Thorn and Truman 1989). In adult *Manduca sexta*, four pairs of nerves exit the posterior of the terminal ganglia and extend to the oviducts. One branch of the nerve VN7 innervates the lateral oviduct, DN8 passes over the lateral oviduct and bifurcates with one branch continuing to the muscles of the body wall and spiracle closer muscle and the other joining with the second branch of VN7. Branches of VN8 innervates the spermatheca, the common oviduct, the ducts of the accessory glands and the bursa copulatrix. The nerve TN8 innervates the accessory glands and the posterior of the common oviduct and also carries the PN (proctodeal nerves) which innervate the hindgut.

Modulation of the myogenic contractions of the insect oviduct is not well understood in most species and particularly not in Lepidopterans. Myogenic contractions act to propel the eggs towards the ovipositor during egg laying and this process is under neurohormonal control (Giradie and Lafon-Cazal 1972, Miller 1975 and Cook *et al.* 1984). Egg laying at inappropriate times is prevented by inhibitory neural control from the central nervous system (in *Carausius morosus* (Thomas and Mesnier 1973), in *Locusta migratoria* (Lange *et al.* 1984b)). Neurally driven contractions of the common oviduct and junctional area act to retain eggs in the lateral oviduct. Central pattern generators (see Delconyn 1980, Grillner 1985) located in the last two abdominal ganglia are activated by descending inhibitory stimulation (in *Calliptamus sp.* and *Decticus albifrons* (Kalogianni and Theophilidis 1995)) and the output from central pattern generators is modulated by neuropeptides (Harris-Warrick and Marder 1991).

In *Periplaneta americana* (Orchard and Lange 1987), *Leucophaea maderae* (Holman and Cook 1985), *Tabanus sulficrons* (Cook and Meola 1978) and *Locusta migratoria* (Lange *et al.* 1986) the neuropeptide proctolin has been implicated in the

myostimulatory regulation of oviduct motility. In addition, the oviducts of *Periplaneta americana* (Orchard and Lange 1987) and *Locusta migratoria* (Orchard and Lange 1985) are innervated by neurones containing the amine, octopamine.

A bioassay using the oviduct of the locust *Locusta migratoria* has been used in the screening for myotropic peptides from the nervous system of this insect. A number of the isolated neuropeptides are myotropic in the oviduct (review: Schoofs *et al.* 1993d). All tested members of the locustamyotropins, locustapyrokinins and locustatachykinins are active on the oviduct of *Locusta migratoria*, as are locustamyoinhibiting peptide, schistoFLRFamide (locustamyosuppressin) (Robb *et al.* 1989), locustamyoinhibin, the locusta-accessory gland myotropins and the peptide CCAP (Dircksen *et al.* 1991). The association of schistoFLRFamide and other members of the FLRFamide family with the locust oviduct has been examined in some depth. SchistoFLRFamide immunopositive axons are present in nerves innervating the oviduct (Schoofs *et al.* 1993a) confirming a neuromodulatory role for these peptides in this muscle. Immunological studies using anti-CCAP antisera have revealed that no CCAP immunopositive axons innervate the oviduct in *Locust migratoria* (Dircksen *et al.* 1991) however these tissues could be affected by circulating CCAP released from the neurohaemal perisympathetic organs.

Less is known about the distribution and function *in vivo* of the other oviduct myoactive peptides and also about muscle regulation in other regions of the female reproductive system.

The function of the spermatheca is to store sperm from the male until required for the fertilisation of the eggs prior to oviposition. The movement of sperm from spermatophore to spermatheca may be mediated by contraction of the genital

duct muscles initiated by pharmacologically active substances from the male accessory glands (Davey 1960). Effective sperm transport depends on the presence of male accessory gland secretion in both *Trichoplusia ni* (Leopold 1976) and *Drosophila melanogaster* (Lefevre and Jonsson 1962) but the process is not well understood. Extracts of the male accessory gland were found to be active in the oviduct (Lafon-Cazal *et al.* 1987) and proctolin (Paeman 1990) and the locusta-accessory gland myotropins I and II (Paeman *et al.* 1991a,b) were isolated from these glands. These peptides may be transferred to the female during copulation and stimulate the reproductive tract muscles to contract so aiding in sperm uptake by the spermatheca of the female. Myotropic factors eluting at the same time as those from male accessory glands from HPLC separations were present in spermatophores but in females were present only in the spermatheca of mated insects (Schoofs *et al.* 1993d).

Contractions of the spermatheca are also required to control the release of stored sperm from the spermatheca at the time of egg fertilisation (Okelo 1979). Kuster and Davey (1986) proposed that this could be brought about by a myotropin acting on the muscles of the spermatheca to induce contractions. Okelo (1979), using electrophysiological experiments in *Schistocerca gregaria* suggested that the presence of the egg in the common oviduct mechanically stimulates receptors which project to the eighth abdominal ganglion. Motor neurones controlling the seminal receptaculum induce contractions leading to the release of stored sperm. Proctolin is myostimulatory in the spermatheca of *Rhodnius prolixus* (Lange 1990) and *Locusta migratoria* (Lange 1993) and a proctolin-like peptide has been shown to be associated with these tissues by immunohistochemistry, HPLC extraction and bioassay but knowledge of further factors modulating this tissue or controls in other species is lacking.

The Male

Studies of the innervation of the male reproductive system of the insect have been limited but, as in the female, the entire tract is usually innervated from the terminal ganglia. In adult *Manduca sexta* (Thorn and Truman 1989) the vas deferens passes through a cleft in the TN8 nerve, the reproductive tract being innervated solely by a branch from TN8 which leaves at this point. The branch bifurcates and one branch innervates the lower ejaculatory duct and penis, the other goes to the upper ejaculatory duct and the accessory glands. The contents of these nerves has not been studied in *Manduca* but proctolin has been immunolocalised in the nerve fibres of the muscle layers around the accessory gland tubules in the locust (Paeman 1991 in Schoofs *et al.* 1993). Locusta-accessory gland myotropin I (Lom-AG-MT-I) immunoreactivity was seen in the muscle layer of the tubules and the ejaculatory duct (Paeman *et al.* 1992). Lom-AG-MT-II immunoreactivity was seen in the muscle layer around the accessory gland tubules (Paeman 1991 in Schoofs *et al.* 1993). Receptors for schistoFLRFamide may be present in the male accessory glands of the locust as this peptide decreases the basal membrane potential of the tubules (Schoofs *et al.* 1993a).

1.3.4 The Aims of the Present Study.

The controls over the rhythm of myogenic contractions in insect visceral muscle are not well understood. Recent advances in peptide isolation and sequencing have resulted in a large number of known insect myotropic neuropeptides being identified and documented. Physiological studies directed towards discovering the *in vivo* function of many of these peptides have been lacking until lately. The ready availability of synthetic peptides greatly simplifies the execution of such

studies.

In this study, a range of known insect neuropeptides are screened against bioassays of the adult and larval hearts and the oviduct of *Manduca sexta.* A number of peptides, isolated from other insect species, although cardioactive in this insect (Reynolds, unpublished) have not been detected in nervous system extracts. The response of the *Manduca* oviduct to myotropic peptides has not previously been reported. Results from this study will aid in determining if members of particular peptide families are represented in this insect and pointing to possible roles *in vivo.* The pattern of innervation of the oviduct is investigated and peptide candidates likely to be involved in the modulation of oviduct motility are determined. A screening of extracts of various nervous system tissues from both *Manduca sexta* and *Schistocerca gregaria* separated by HPLC, results in the detection of a number of myotropic fractions.

Chapter 2

The Actions of Bioactive Peptides in the Heart of *Manduca sexta.*

2.1 Introduction

In recent years major improvements have occurred both in peptide isolation and in structural characterisation techniques. This has led to a rapid increase in the numbers of insect neuropeptides which have been identified and sequenced from a range of species. Initially many of the identified insect neuropeptide sequences have been from Orthoptera (locusts and cockroaches) and far fewer from the more economically important groups such as Lepidoptera (moths).

The identification and subsequent isolation of a peptide is only made possible if a reliable method of detection can be determined. Myoactive peptides have proven to be particularly amenable to purification, because their ability to induce or modulate contractions of visceral and skeletal muscles can be exploited by the use of an *in vitro* bioassay system. The screening of fractions from nervous system extracts against the bioassay enables myoactive factors to be detected and followed throughout the isolation procedure as fractions containing myotropins

active on the specific muscle will alter the spontaneous contraction rate.

Muscle bioassays have proved to be simple and reliable detection method for myotropins and several bioassay techniques have been developed and used successfully for this purpose. The majority of studies have however centered on factors active in the hindgut of the cockroach, *Leucophaea maderae* (review: Holman *et al.* 1991). For example, in *Leucophaea maderae* 12 novel myoactive peptides have been isolated and sequenced (Holman *et al.* 1990). In the house cricket *Acheta domesticus*, five myoactive peptides have been characterised (Holman *et al.* 1990b) and from *Locusta migratoria* 21 different myotropins have been identified (review: Schoofs *et al.* 1993d). Myotropins have also been isolated from the flesh fly, *Neobellieria bullata* (Fonagy *et al.* 1992b,c) and the Colorado beetle, *Leptinotarsa decemlineata* (Spittaels *et al.* 1991) using the cockroach hindgut bioassay. The extensive and almost exclusive use of this single bioassay has meant that rather less is known about the activity of these peptides on the insect heart or other visceral muscles, either in homologous or heterologous bioassays. In addition, the possible presence of myotropic neuropeptides not myoactive in the *Leucophaea* hindgut and therefore not previously detected has not been thoroughly investigated.

Work using Lepidopteran species such as *Manduca sexta* has, by contrast to species such as *Locusta*, yielded far fewer myoactive peptides. The heart of *Manduca sexta* provides a useful tool in the search for cardioactive peptides in this insect. Both the adult and larval heart bioassays possess the key features required for a workable detection and isolation method. They are quick and simple to prepare, have a rather stable basal contraction rate, are sensitive to small amounts of cardioactive material, are long-lived and give consistent results both during testing and between preparations.

Whilst not as prolific in myoactive peptides as other species, several neuropeptides have been isolated from the central nervous system of *Manduca sexta* using the heart bioassay of this insect. The presence of these endogenous cardioactive peptides was shown both in caterpillars (Platt and Reynolds 1985) and in adult moths (Tublitz and Truman 1985a).

Initial work on the peptides revealed that the larval and adult stages of development each contained two distinct sets of peptides. In caterpillars these peptides were originally named CAF 1 and CAF 2, and in moths CAP 1 and CAP 2. In further work, it was established that whilst CAF 2 is identical to CAP 2, CAF 1 and CAP 1 can be separated and must therefore be two different peptides (Tublitz *et al.* 1992).

Studies of the physiological activities of these peptides have revealed several rôles. In adult moths the CAPs act as neurohormones and modulate the myogenic heartbeat immediately after adult emergence from the pupal case to facilitate wing inflation (Tublitz and Truman 1985 a,b; Tublitz and Evans 1986) and during flight to increase the circulation of haemolymph between abdomen and thorax (Tublitz 1989). In the latter stages of the embryo the release of CAP 2 stimulates the initial contractions of the hindgut (Broadie *et al.* 1990). CAP 2 is also released in 5th instar larvae and regulates the clearing of the alimentary canal prior to metamorphosis (Tublitz *et al.* 1992).

The CAP 2 fraction has been separated. The previously named CAP 2 actually consists of three distinct peptides (Loi *et al.* 1992) of which only one, CAP 2a has been isolated and sequenced. The sequence of CAP 2a is identical that of crustacean cardioactive peptide (CCAP) ((Hildebrand *et al.* 1990, Cheung *et al.* 1992, Lehman *et al.* 1993). The CAP 1 peptides have as yet not been identified.

The CAPs have to date been the only myoactive peptides isolated from *Manduca* with the use of the *Manduca* heart bioassay. A number of other neuropeptides have been identified and some subsequently sequenced from *Manduca* using other detection methods. The sequences of these are shown in Table 2.1. The peptides corazonin (Veenstra 1991), *Manduca sexta* FLRFamide (Mas-FLRFamide) (Kingan *et al.* 1990) and two extended FLRFamides (Witten *et al.* 1993) have been detected in adult moths with the use of ELISAs. *Manduca sexta* allatotropin (Mas-AT) (Kataoka *et al.* 1989) and *Manduca sexta* allatostatin (Mas-AS) (Kramer *et al.* 1991) have been isolated from the adult moth based on their ability to effect juvenile hormone secretion by the corpora allata of adult moths. Whilst Mas-AT has some cardioacceleratory properties (Veenstra *et al.* 1994), Mas-AS does not show any activity in the heart of *Manduca sexta* (D. A. Schooley, personal communication to S. E. Reynolds). A number of myoactive fractions have also been detected and two peptides have been isolated and sequenced from larval *Manduca* midgut extracts (Yi *et al.* 1992, Yi *et al.* 1995) using a heterologous *Locusta migratoria* oviduct bioassay.

The number of myoactive peptides from nervous tissue extracts of *Manduca* is unexpectedly low in comparison to species such as *Locusta.* Two explanations are possible; either this insect does not contain as large a number of such peptides or they occur at considerably lower levels undetectable by bioassays and are yet to be found by immunological methods. If *Locusta* requires such a wide range of myoactive peptides for control of physiological processes why should *Manduca* have so few ? The imbalance is also surprising in view of the large size of insect *Manduca* relative to other species and therefore the likely relative abundance of neurological material and ease of dissection. It would seem unlikely that a lack of nervous tissue could explain the differences.

The physiological significance *in vivo* of peptides isolated from *Manduca* has yet to be fully explored. Past work on the heart of *Manduca sexta* has revealed a limited amount of information regarding the peptide pharmacology of the heart of this insect.

The aim of this study was to test a range of neuropeptides both from *Manduca* and from other species for cardioactivity in the semi-isolated heart of *Manduca.* The results would aid in the search for further myotropic peptides in this insect by identifying the receptors present in the heart and therefore, the peptide families which are likely to be represented in *Manduca.* The study also aimed to provide information regarding activity and responses of this bioassay to various known neuropeptides. This would be of use in determining the physiological control mechanisms involved in regulation of the heartbeat. A comparison of the responses of both the adult and larval stages to various neuropeptides would aid in the identification of likely candidates for the as yet unidentified cardioactive peptides in the CAP2, the CAP1 and the CAF1 fractions.

2.2 Methods

2.2.1 The Experimental Insect

The stages of tobacco hornworm used throughout this study were taken from a laboratory culture of *Manduca sexta*, maintained on an artificial diet according to Bell and Joachim (1976) and kept at 25°C, 50-60% relative humidity under a long day photoperiodic regime (L:D, 17:7).

CAP 1a	sequence not available
CAP 1b	sequence not available
CAP 2a (CCAP)	Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly- Cys-NH_2
CAP 2b	sequence not available
CAP 2c	sequence not available
CAF 1	sequence not available
Corazonin	pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn- NH_2
Manduca FLRFamide	pGlu-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2
Manduca allatotropin	H-Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly- Phe-NH_2
Manduca allatostatin	pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile- Ser-Cys-Phe-OH
Manduca midgut myotropin I	Ala-Glu-Pro-Tyr-Thr-NH_2
Manduca midgut myotropin II	Asp-Ile-Pro-Pro-Arg-NH_2

Table 2.1: Table showing myoactive neuropeptides and sequences, where known, identified in *Manduca sexta*

2.2.2 Preparation of Larval Heart Bioassay

The larval heart bioassay (see Platt and Reynolds 1985) was performed using fifth instar larvae at 12 hours before wandering. The larvae were not sexed prior to use.

Larvae were anaesthetised by immersion in distilled water at room temperature for 20 minutes. The animal was then opened ventrally and the body wall pinned out onto a Sylgard- (Dow Corning) filled dish. The preparation was positioned over a cavity cut out of the Sylgard thus allowing air to circulate to the spiracles. The gut and the abdominal nerve cord were removed and the preparation washed thoroughly in several changes of *Manduca* saline (for composition see Appendix). The dish was positioned at approximately 25° to the horizontal using a retort stand and clamps. A flow of saline, maintained by gravity feed, was continuously perfused onto the caudal end of the heart at a rate of the approximately 40ml hour $^{-1}$.

The preparation was left with a saline flow for 30 minutes, after which time a steady heartbeat was usually observed. Preparations with no or only a weak pulse were discarded at this point.

In viable preparations, a small hook (formed from a 1AA gauge entomological pin) was inserted under the heart avoiding damage to the tissues and snagging of the body wall muscle. The hook was connected via a length of cotton to the arm of a Palmer isotonic force transducer, counterbalanced with a 140mg plasticine weight. Output from the transducer was recorded by a flat bed potentiometric chart recorder (Houston Omniscribe).

A steady heartbeat could more readily be obtained if the heart was cut approximately 3mm above the hook. This also removed the influence of multiple pacemakers along the heart. In a small number of preparations it was also necessary to make a cut at a similar distance below the hook.

Application of test substances was usually carried out on hearts with a rate of 10-12 beats minute^{-1}.

2.2.3 Preparation of Adult Heart Bioassay

The adult heart bioassay was performed using pharate adult females within 12 hours of emergence (stage 7, according to Samuels and Reynolds (1993)).

The head and thorax were removed and the abdomen opened ventrally, and pinned onto a sylgard dish. The reproductive organs, gut and abdominal nerve cord were removed. The preparation was then washed thoroughly in saline. A short section of the caudal end of the heart was lifted (approximately 1mm) and connected via a cotton thread to an isotonic force transducer. Other details of saline perfusion and testing are similar to those of the larval heart bioassay.

Testing was usually carried out on preparations with a heartrate of 12-14 beats minute^{-1}.

2.2.4 Experimental Procedure

Fresh test samples of peptide solutions were prepared daily from aliquots of 10^{-5}M of the peptide in distilled water stored at -40°C. Serial dilutions were prepared

in either fresh adult or larval *Manduca* saline.

Pulse applications of 20μl of test solution were made into the saline flow onto the cut point of the heart using a Gilson pipette. The volume of 20μl was selected with reference to both the limits of detection of the bioassays and the necessity of using the smallest possible amounts of peptide. Figure 2.1 shows applications of various volumes of a 10^{-9} M solution of CCAP to the larval heart. The volume of 20μl gives a response of slightly over half of the maximum response and therefore is within the range detected by this bioassay.

Further applications of samples were only made after the heartrate had returned to that which it was prior to the previous application. Saline control applications were made throughout testing and standard doses of CCAP were used to determine the relative responsiveness of each preparation.

The doses of peptide given are indicated as the concentration (M) of the peptide solution applied to the heart. The actual amount of peptide contained in the 20μl of test solution applied is shown in parentheses.

All assays were performed at room temperature (22$\pm$2°C). Application of test substances was not started until the heart bioassay had reached equilibrium which was usually 1-2 hours after setting up.

The response of the heart was quantified by comparison of the heartrate during the first minute following application with that of the minute preceding application.

A comprehensive screening of all the known insect myoactive neuropeptides would

be beyond the scope of this project and a selection of peptides was made based on structure and availability.

Peptides used were:-

- Crustacean cardioactive peptide (CCAP) (synthesised for this study)
- Cardioactive peptide 2s – a,b and c (gift of N. Tublitz, University of Oregon, USA.)
- *Manduca sexta* allatotropin (Mas-AT) (Sigma)
- Locustatachykinin II (Lom-TK-II) (synthesised for this study)
- FMRFamide (Sigma)
- FLRFamide (Sigma)
- LPLRFamide (Peninsula)
- SchistoFLRFamide (gift of P. D. Evans, University of Cambridge.)
- ManducaFLRFamide (gift of Dr T. Kingan, USDA, Beltsville, Maryland, U.S.A.)
- CalliFMRFamides- 1,3 and 5 (gift of H. Duve and A. Thorpe, Queen Mary and Westfield College, University of London.)
- Leucomyosuppressin (Lem-MS) (Peninsula)
- Corazonin (Sigma)
- Leucopyrokinin (Lem-PK) (Sigma)
- *Heliothis zea* Pheromone biosynthesis acivating neuropeptide (Hez-PBAN) (Peninsula)
- Proctolin (Sigma)
- Callatostatins - 1 and 5 (gift of H. Duve and A. Thorpe, Queen Mary and Westfield College, University of London.)

2.3 Results

A range of neuropeptides were presented to the semi-isolated heart preparations of both larvae and adults. Of those tested a number were found to be cardiostimulatory, some had no effect on the heartrate and four were cardioinhibitory.

The results from all the peptides on the two heart bioassays are presented in Table 2.2 giving the reponse and the minimum dose at which cardioactivity could be detected.

The response of both the larval and adult hearts to all the cardioactive peptides was concentration-related therefore a dose response curve could be constructed for each peptide. These are shown in graph form. Each graph shows the data from one heart preparation. Each point represents the response obtained from one application to one insect preparation. The line represents the mean heartrate change calculated from three applications of the same concentration.

The application of a cardioactive sample caused both chronotropic and inotropic effects. These were observed as rate and amplitude changes to the spontaneous contraction rate. For the purposes of this study, only the chronotropic effects were considered to give useable data for a number of reasons. Most adult hearts and some larval hearts did not respond with changes in amplitude but only with rate responses. In those preparations which did show inotropic effects, the responses were usually not comparable between hearts and large variations were observed between the extent of the responses from preparation to preparation. At higher concentrations of cardioactive peptide the inotropic response diminished because the heart failed to relax completely between beats. For these reasons the inotropic effects on heartrate did not give an accurate and reliable representation of the

responses of the hearts and were not used in this study.

At responses in excess of 300% of the basal heartrate, systolic arrest of the heart usually occured. The heart beat became too rapid for the heart to relax following each contraction and a flat line was obtained on the chart recorder. Similarly, high concentrations of cardiosuppressive peptides caused a complete cessation of the heartbeat making heartrate calculation impossible. High concentrations of cardioactive peptides also caused prolonged effects on the basal heartrate necessitating extended washing after each application before return to the basal rate. Hence higher concentrations of the most active cardioactive peptides were impossible to test.

Crustacean Cardioactive Peptide (CCAP)

CCAP was the most potent cardiostimulatory peptide tested in both the adult and the larval hearts. The dose response curves are shown in Fig. 2.2 and Fig. 2.3. The larval heart preparation was found to be more sensitive than the adult heart. Threshold concentrations were between 5×10^{-11} M and 10^{-10} M (1 to 2 fmol) in the adult heart and between 10^{-12} M and 10^{-11} M (0.02 to 0.2 fmol) in the larva. In both the adult and the larva the concentration giving maximum response could not be deduced because systolic arrest occured at concentrations of CCAP above 10^{-8} M (200 fmol) in the adult and 10^{-9} M (20 fmol) in the larva. The concentration of CCAP which gives a 50% response could, therefore, not be calculated. At 10^{-8} M the adult heart gave a response 333.0% above the base rate. The larval heartrate increased to 322.2% above base rate at a concentration of 10^{-9} M (20 fmol).

	Activity in	Adult Heart	Activity in	Larval Heart
Peptide	Threshold Dose	Dose giving 3× basal rate	Threshold Dose	Dose giving 3× basal rate
CCAP	10^{-10} M	10^{-9} M	10^{-11} M	7.5×10^{-10}
Mas-AT	10^{-8} M	2×10^{-7} M	inactive	
Lom-TK II	inactive		10^{-10} M	7.5×10^{-8} M
FMRFamide	inactive		inactive	
FLRFamide	inactive		inactive	
LPLRFamide	inactive		10^{-7} M	ND
Mas-FLRFamide	10^{-8} M	7×10^{-8} M	10^{-9} M	ND
SchistoFLRFamide	10^{-9} M	ND	10^{-9}M	ND
Lem-MS	inactive		10^{-7} M	ND
CalliFMRFamides				
1	not tested		10^{-7} M	ND
3	not tested		10^{-7} M	ND
5	not tested		10^{-8} M	ND
Corazonin	10^{-10} M	2×10^{-8} M	10^{-10} M	2.5×10^{-8} M
Hez-PBAN	inactive		10^{-6} M	ND
Lem-PK	inactive		5×10^{-7} M	ND
Proctolin	inactive		inactive	
Callatostatins				
1	not tested		10^{-6} M	ND
5	not tested		10^{-8} M	ND

Table 2.2: Table showing cardioactivities of various known neuropeptides in the adult and larval hearts of *Manduca sexta*. ND – At the doses tested, the heartrate changed by less than three times the basal rate so no value is shown here.

Isolated peptides from the CAP2 fraction were tested on both the adult and larval hearts, the results are shown in Figures 2.4 and 2.5. Limited experimental material restricted testing to a single application at each nerve cord equivalent. In the adult CAP2c was the most cardioactive of these peptides with a maximum response of 123.0% rate increase at a concentration of 0.05 nerve cord equivalents the response dropped to a 63.0% increase at 0.1 nerve cord equivalents. CAP2b was the next most cardioactive with a maximum response of 76.0% rate increase at 0.1 nerve cord equivalents. CAP2a was the least cardioactive of these peptides with the greatest response at the maximum concentration tested of 0.1 nerve cord equivalents with a rate increase of 35.0%. CCAP applied to the same heart bioassay gave a similar dose- response curve to CAP2a when applied at 10^{-11}, 5 $\times$ 10^{-11} and 10^{-10} M.

In the larval heart CAP2a was the most cardioactive with a maximum response of 50.0% rate increase at 0.1 nerve cord equivalents over the range tested. CAP2c was the next most active and with a rate increase of 22.0% at 0.1 nerve cord equivalents and CAP2b was the least active with a rate increase of 11.0% at 0.1 nerve cord equivalents. Synthetic CCAP gave a similar dose response curve to that of CAP2a when applied at 10^{-11}, 5 $\times$ 10^{-11} and 10^{-10} M.

In summary, the CAP2b and c peptides cause a heart rate increase in the adult of approximately six to seven times that in the larval heart at equivalent doses. In the larval heart, CAP2a gives a rate increase of approximately one and a half times that in the adult heart.

Manduca sexta Allatotropin (Mas-AT)

Mas-AT was active only in the adult heart with no response up to a concentration of 10^{-6} M (20 pmol) in the larval heart. The dose response curve is shown in Fig. 2.6. The threshold concentration in the adult heart was between 5×10^{-9} M and 10^{-8} M (100 fmol to 200 fmol) with 10^{-6} M giving a response of 316.7% increase over the basal heartrate.

Locustatachykinin II (Lom-TK II)

The peptide locustatachykinin II was not cardioactive in the adult heart up to a concentration of 10^{-6} M but was active in the larval heart. The dose response curve is shown in Fig. 2.7. In the larval heart the threshold was between 10^{-11} M and 10^{-10} M (0.2 to 2 fmol). The maximal response of a 290.0% increase over base rate was obtained at 10^{-6} M.

FMRFamide and related peptides

The peptides FMRFamide and FLRFamide were not active on either the adult or the larval hearts up to a concentration of 10^{-6} M.

The peptide LPLRFamide was inhibitory in the larval heart with a threshold of between 10^{-8} M and 10^{-7} M (200 fmol to 2 pmol). The maximal response of 32.2% inhibition occurred at 10^{-6} M. The response of the larval heart was a rapid cessation of the beat after application, a quiescent period with the duration depending on the concentration applied and a rapid recovery to the basal rate

(see Fig. 2.18). This peptide was inactive in the adult heart.

The peptides schistoFLRFamide and manducaFLRFamide were cardioactive in both adults and larvae. The concentration response curves are shown in Fig. 2.9 and Fig. 2.10. In adults the threshold for schistoFLRFamide was between 10^{-10} M and 10^{-9} M (2 to 20 fmol) and between 10^{-9} and 10^{-8} M (20 fmol to 200 fmol) for manducaFLRFamide. The maximal response obtained for schistoFLRFamide in the adult was 81.8% increase with a concentration of 10^{-6} M. ManducaFLR-Famide was more active and at 10^{-6} M gave a response of just over 277.8% increase over the basal rate.

In larvae the activity of both schistoFLRFamide and manducaFLRFamide were similar with thresholds of between 10^{-10} and 10^{-9} M (2 to 20 fmol). SchistoFLR-Famide gave a mean maximum response of 90.9% increase over the base rate at 10^{-6} M. ManducaFLRFamide gave an increase of 141.0% at 10^{-6} M.

Leucomyosuppressin (Lem-MS) was cardioinhibitory in the larval heart, with a threshold of between 10^{-8} M and 10^{-7} M (200 fmol to 2 pmol) (see Fig. 2.11). Maximal inhibition occurred at 10^{-6} M with a 15.3% decrease in heartrate. The response of the larval heart to this peptide was biphasic with an initial brief rate increase followed by inhibition and gradual recovery to the basal rate (see Fig. 2.18). Lem-MS was inactive in the adult heart.

Three further members of the FMRFamides family isolated from the blowfly, *Calliphora vomitoria* were less active than either schistoFLRFamide or manduca-FLRFamide when tested in the larval heart. The dose response curves relating to the calliFMRFamides are shown in Fig. 2.12. The calliFMRFamides 1 and 3 had threshold values of between 10^{-8} and 10^{-7} M (200 fmol to 2 pmol) and

calliFMRFamide 5 a threshold of 10^{-9} to 10^{-8} M (20 fmol to 200 fmol). The maximum response at 10^{-6} M did not exceed 16% for any of these peptides. The differences in the threshold and maximum responses for these three peptides are not significantly different for this number of samples.

Corazonin

Corazonin was cardiostimulatory in both the adult and larva. The dose response curve for this peptide is shown in Fig. 2.13 and Fig. 2.14. Threshold concentrations were between 10^{-11} M and 10^{-10} M (0.2 to 2 fmol) in both stages. The dose response curves for both stages were similar. No obvious maximum response was seen, therefore the concentration at which 50% response occurs cannot be deduced. In the adult, at a concentration of 10^{-6} M the heartrate increased to 291.0% of the base rate and to 301.9% at a concentration of 5×10^{-7} M in the larva.

Heliothis zea Pheromone Biosynthesis Activating Neuropeptide (Hez-PBAN)

The peptide Hez-PBAN was not active in the adult heart up to a concentration of 10^{-6} M. The dose response curve relating to the larval response is shown in Fig. 2.15. Only the larval heart was responsive to this peptide with a threshold of 10^{-7} M to 10^{-6} M (2 to 20 pmol). At 10^{-6} M the response was 26.8% over the base rate.

Leucopyrokinin (Lem-PK)

The adult heart was not responsive to Lem-PK up to a concentration of 10^{-6} M. The dose response curve for cardioactivity of LPK in the larval heart is shown in Fig. 2.16 with a threshold of 10^{-8} M to 5×10^{-7} M (200 fmol to 10 pmol). The maximum response at 10^{-6} M was 167.4% over the base rate.

Proctolin

Proctolin was not cardioactive in either the adult or larval hearts up to a concentration of 10^{-6} M (20 pmol).

Callatostatins

The callatostatins inhibited the beating of the larval heart. The dose response curves are shown in Fig. 2.17. Callatostatin 5 was cardioinhibitory at a threshold concentration of between 10^{-9} M and 10^{-8} M (20 to 200 fmol) and a maximal response of 82.4% was reached at 10^{-6} M. Callatostatin 1 was active at a threshold of 10^{-7} M to 10^{-6} M (2 to 20 pmol) with a response of 57.9% reached at 10^{-6} M. The pattern of response to both peptides was a gradual slowing and then recovery (see Fig. 2.18).

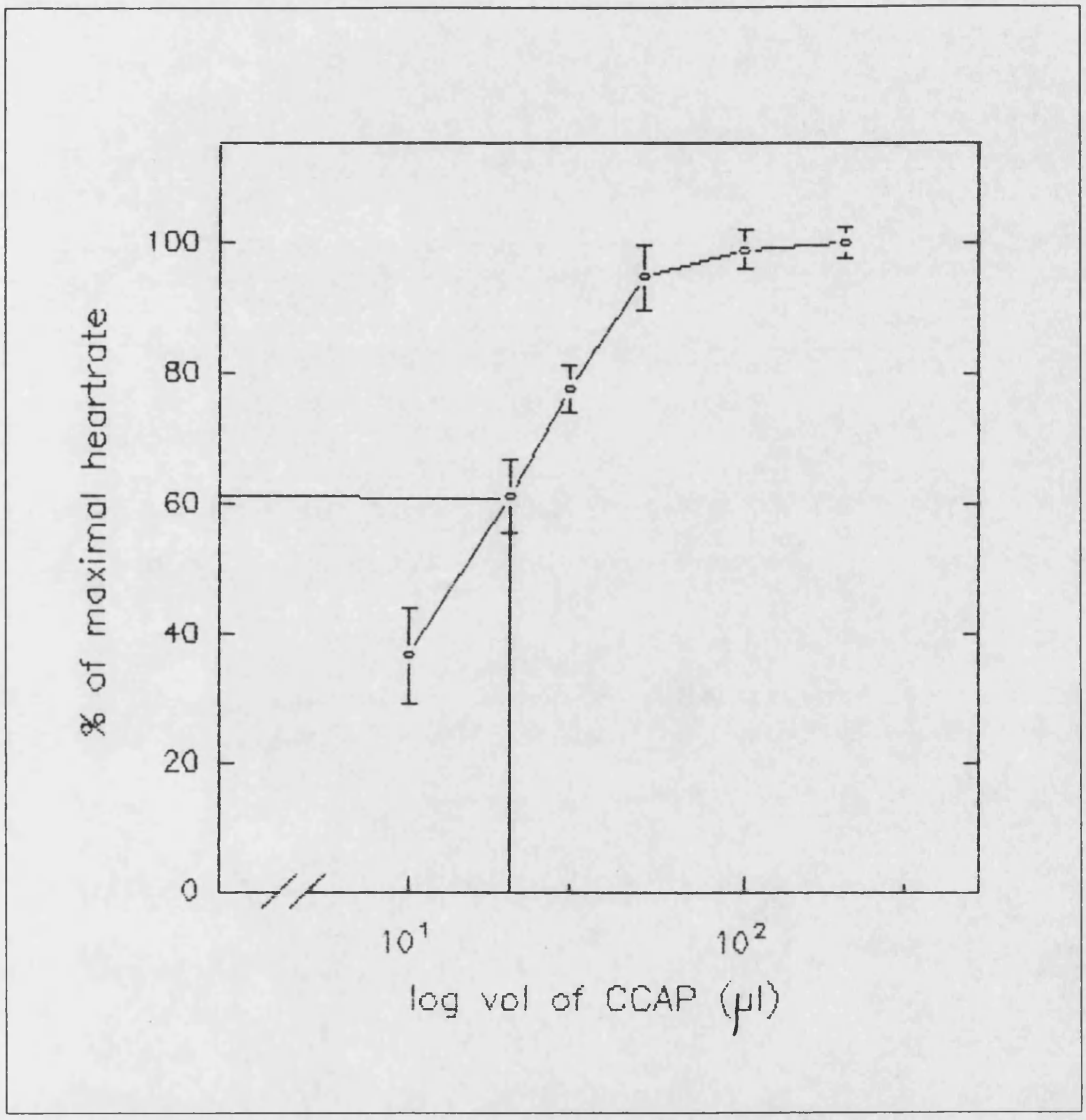

Figure 2.1: Graph showing the response of the larval heart to a range of volumes of 10^{-9} M synthetic CCAP.
Five applications were made of each volume with the point representing the mean response and the error bars representing the 95% confidence limit. The volume of 20µl as used throughout the experiments in this study is marked.

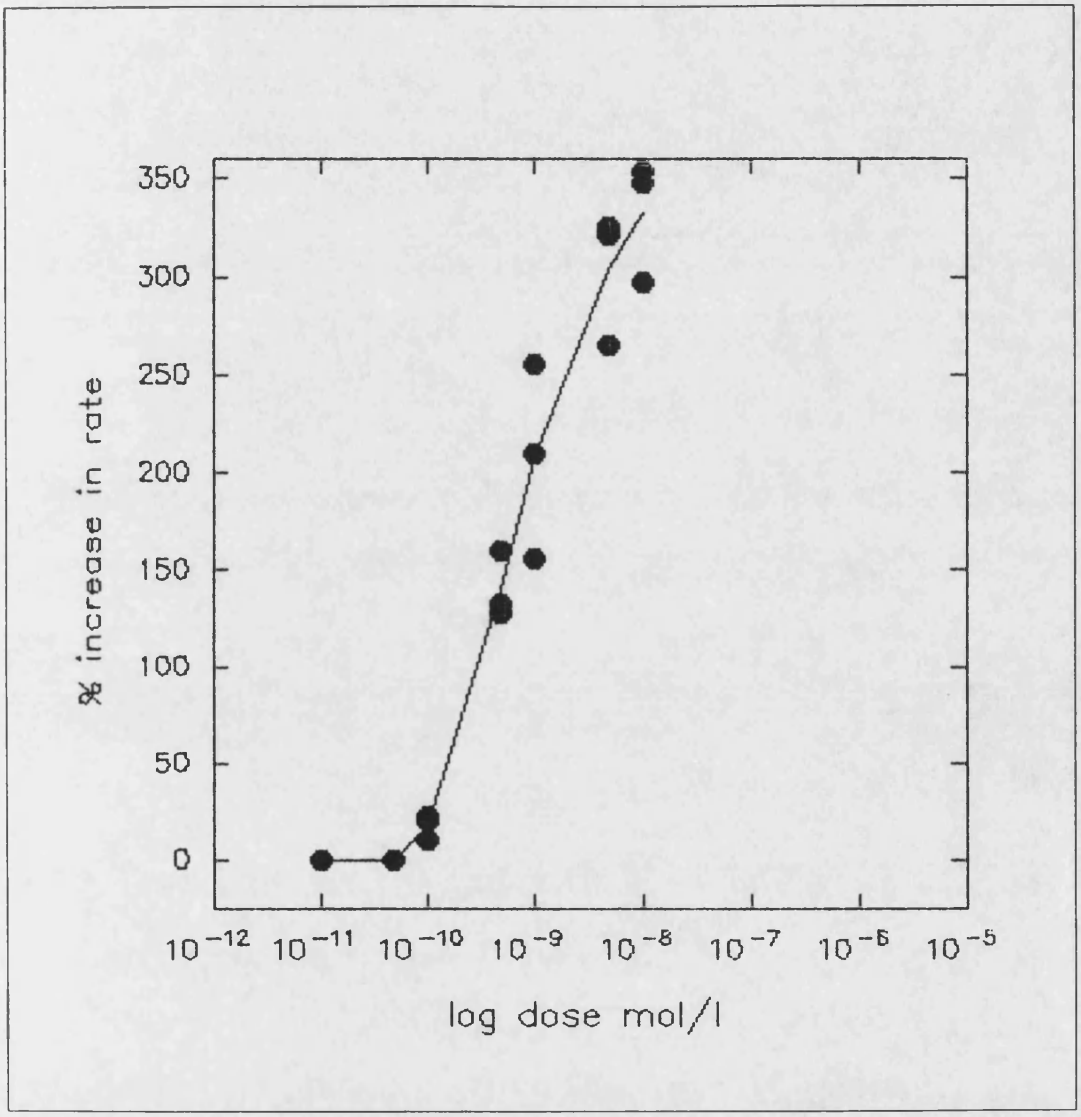

Figure 2.2: Graph showing the response of the adult heart to synthetic CCAP (crustacean cardioactive peptide).
The points represent the result of a single application of the specified concentration and the curve shows the mean of three applications. The data is taken from one heart preparation. All applications were made in a volume of 20μl of adult saline.

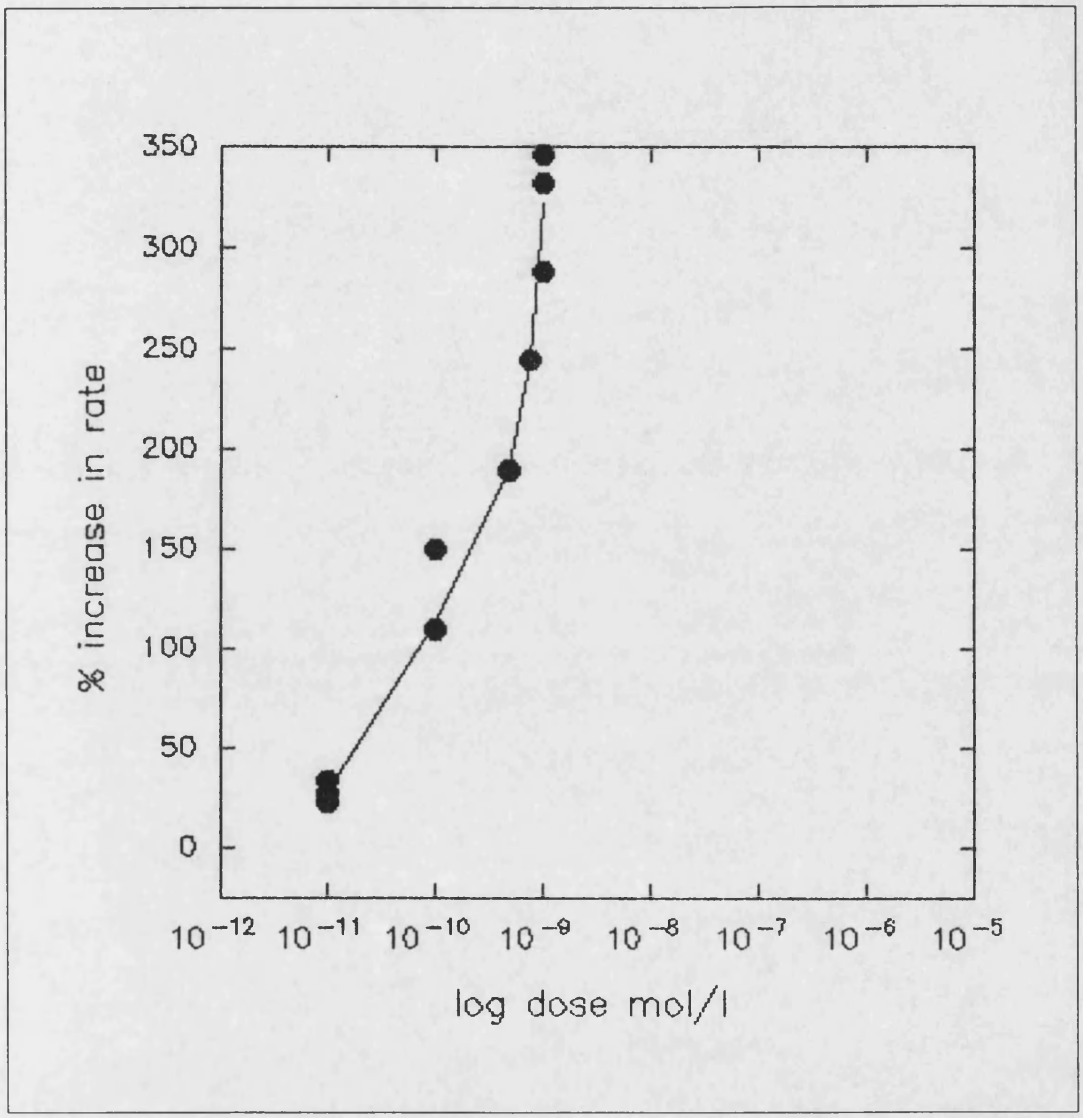

Figure 2.3: Graph showing the response of the larval heart to CCAP.
Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.

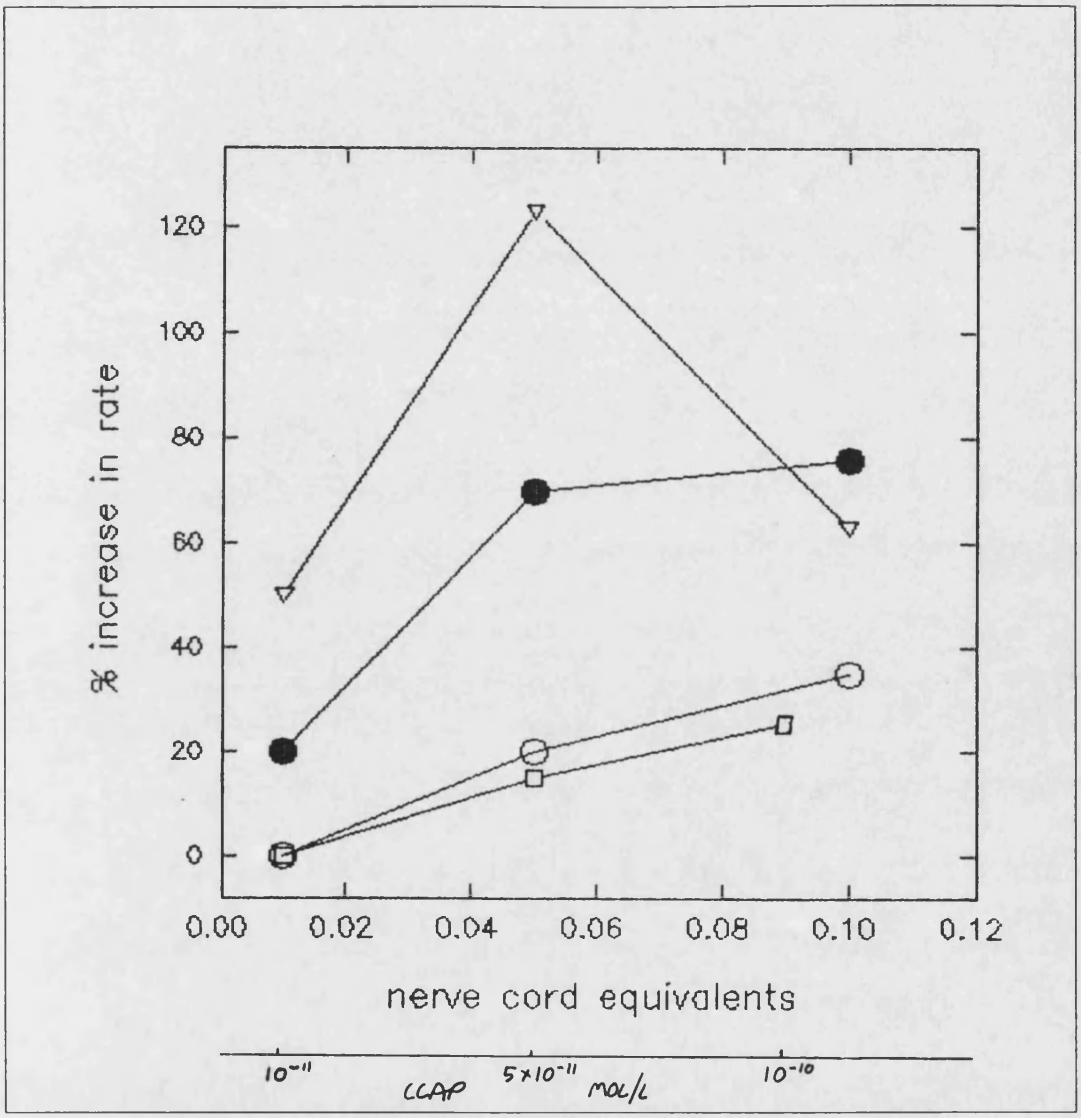

Figure 2.4: Graph showing the response of the adult heart to the three cardioacceleratory peptide 2s.
CAP 2a –o–, CAP 2b –•–, CAP 2c – ▽– and to synthetic CCAP –□–. Each point represents a single application of the specified nerve cord equivalent. Further investigation was not possible due to a lack of experimental material.

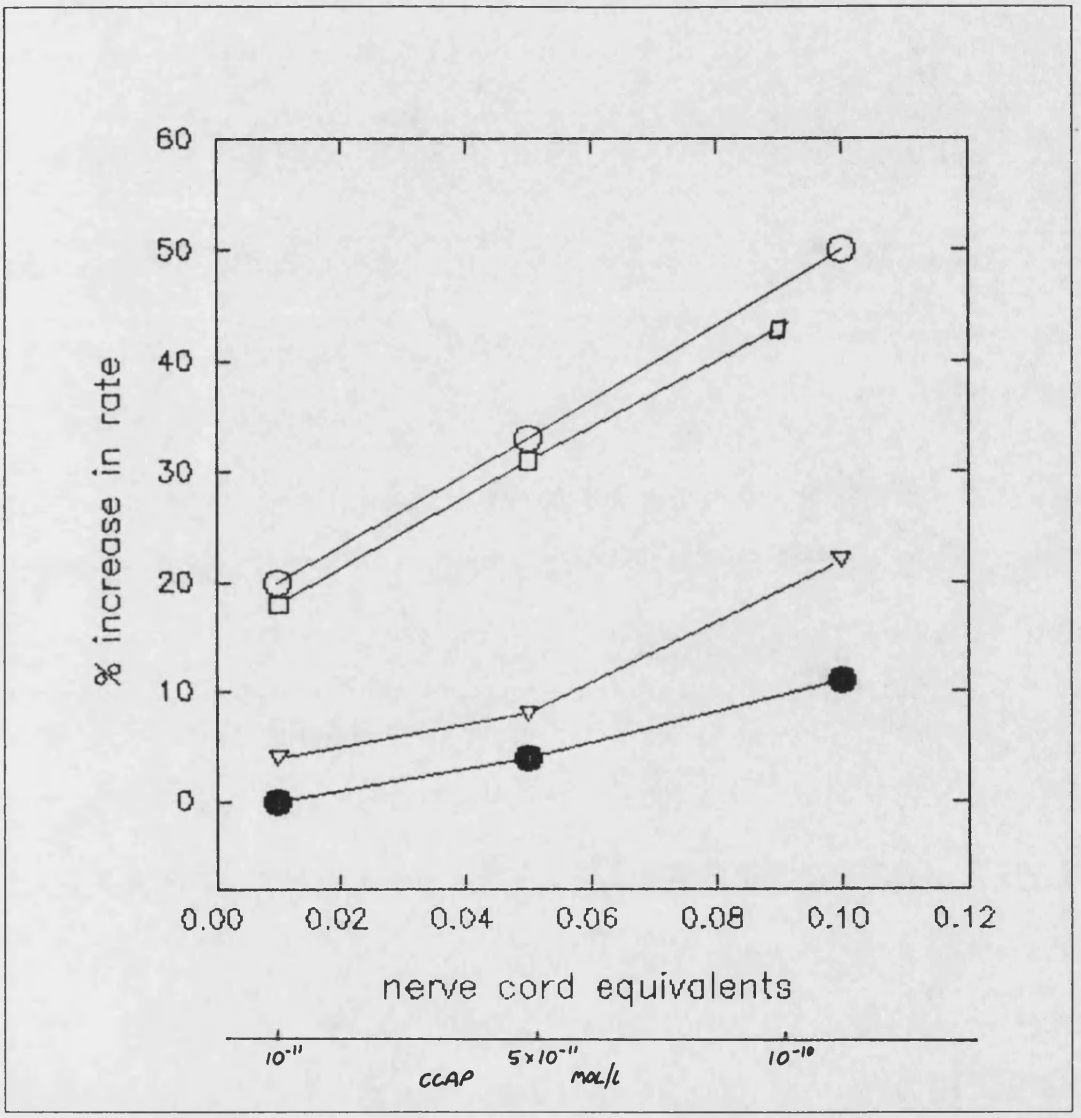

Figure 2.5: Graph showing the response of the larval heart to the three cardioacceleratory peptide 2s.
CAP 2a –○–, CAP 2b –●–, CAP 2c – ▽– and to synthetic CCAP –□–. Each point represents a single application of the specified nerve cord equivalent. Further investigation was not possible due to a lack of experimental material.

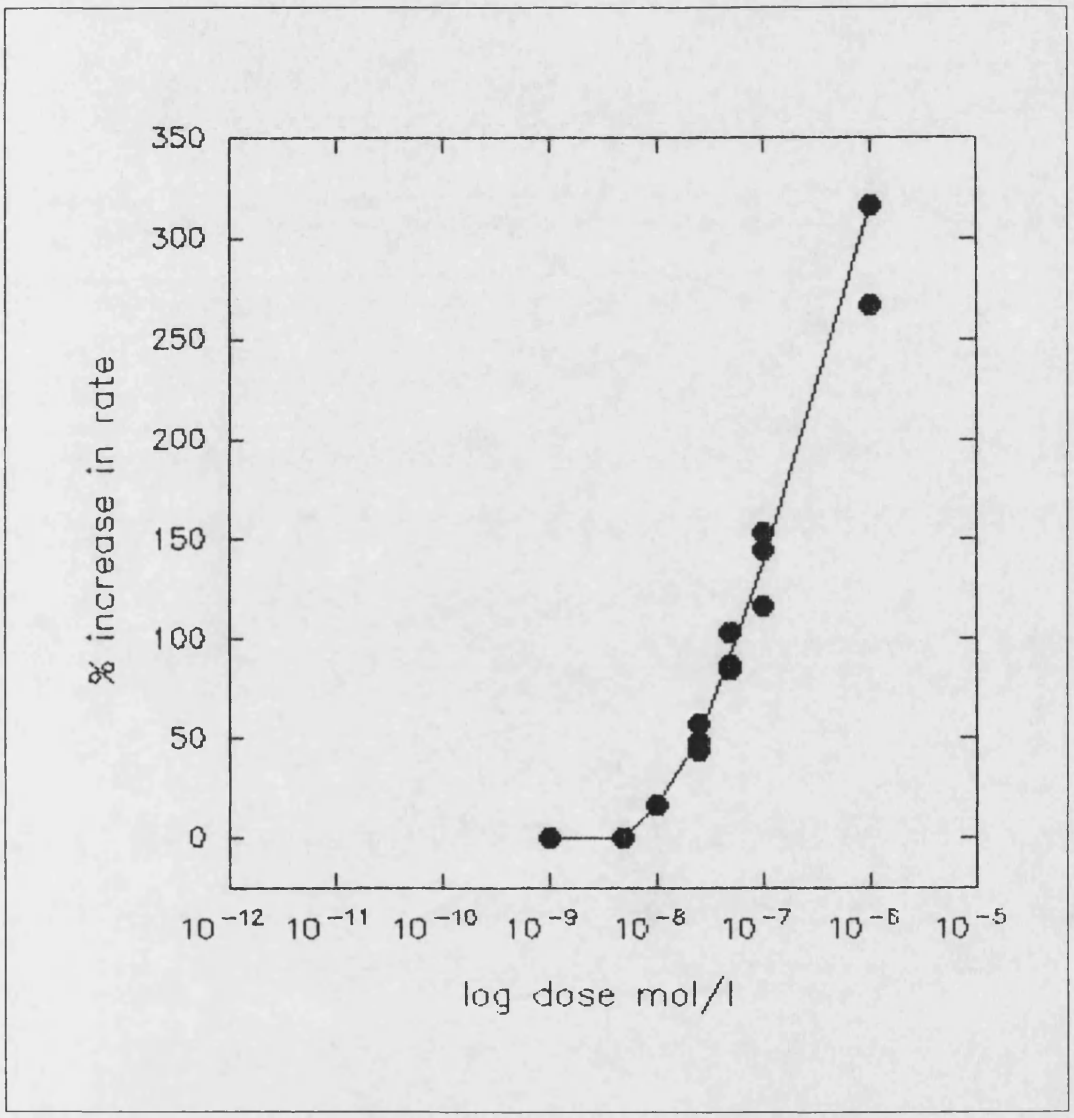

Figure 2.6: Graph showing the response of the adult heart to *Manduca sexta* allatotropin.
Conditions identical to those of the adult heart response to CCAP.

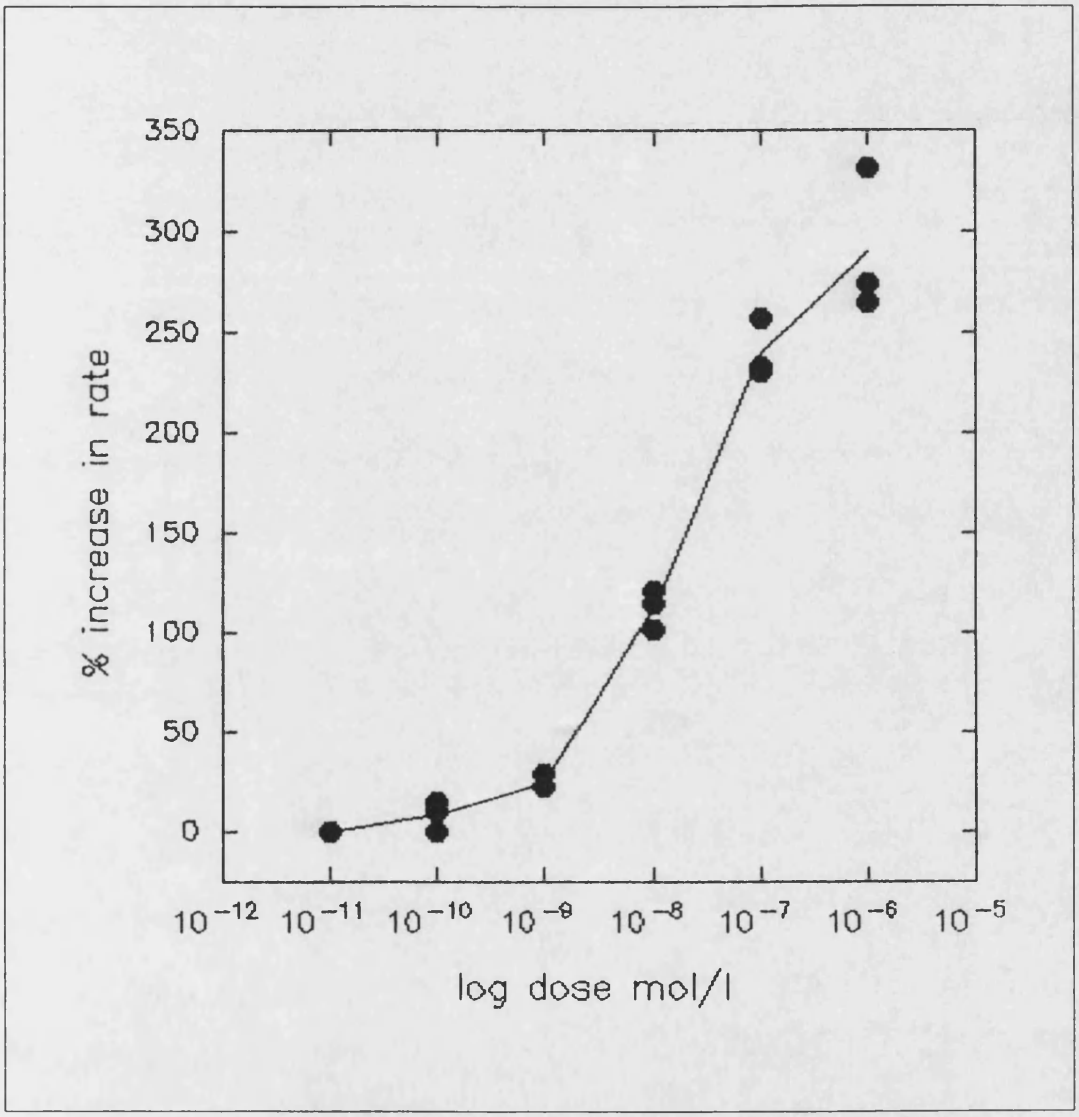

Figure 2.7: Graph showing the response of the larval heart to locustatachykinin II. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

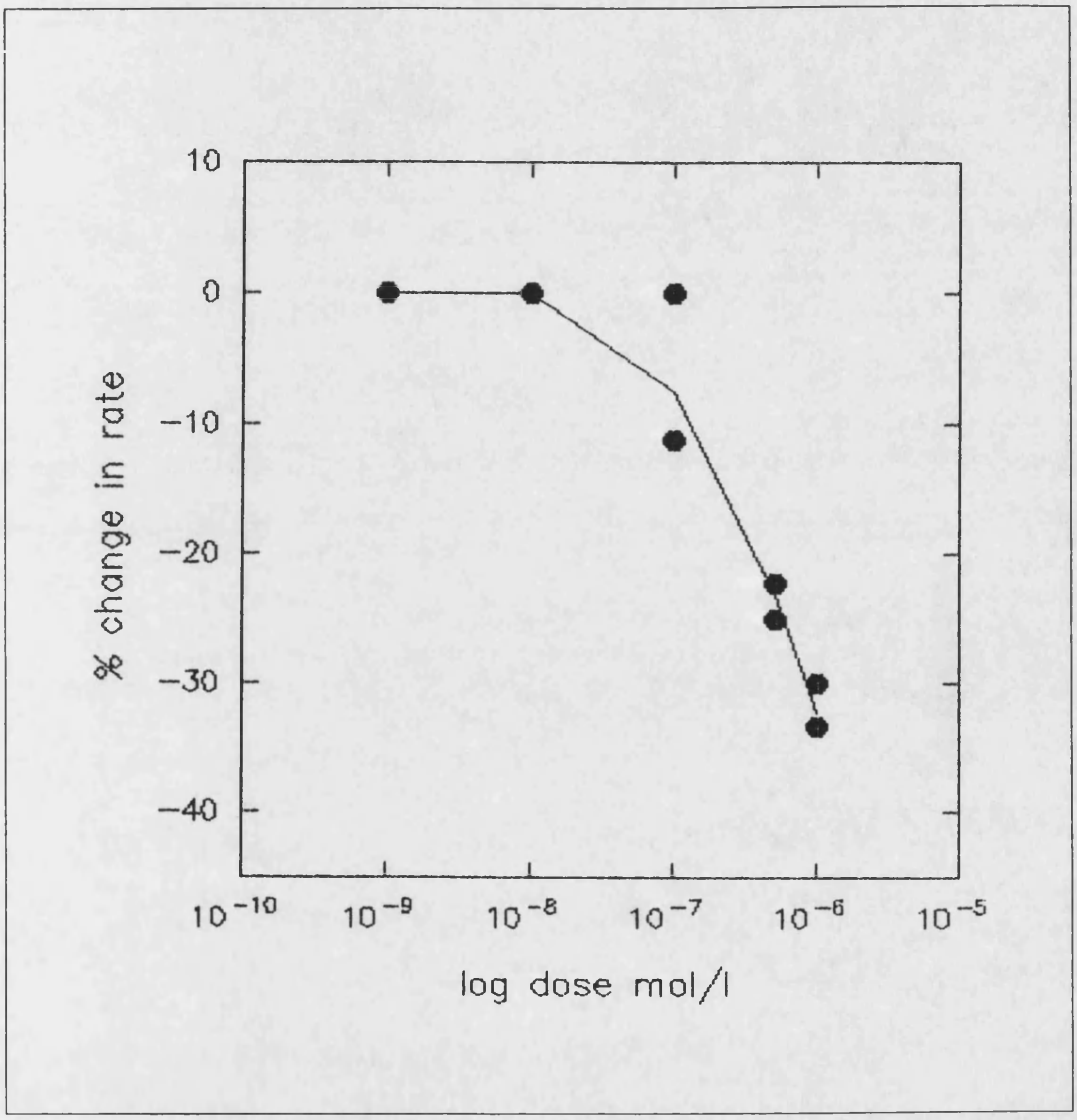

Figure 2.8: Graph showing the response of the larval heart to LPLRFamide. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

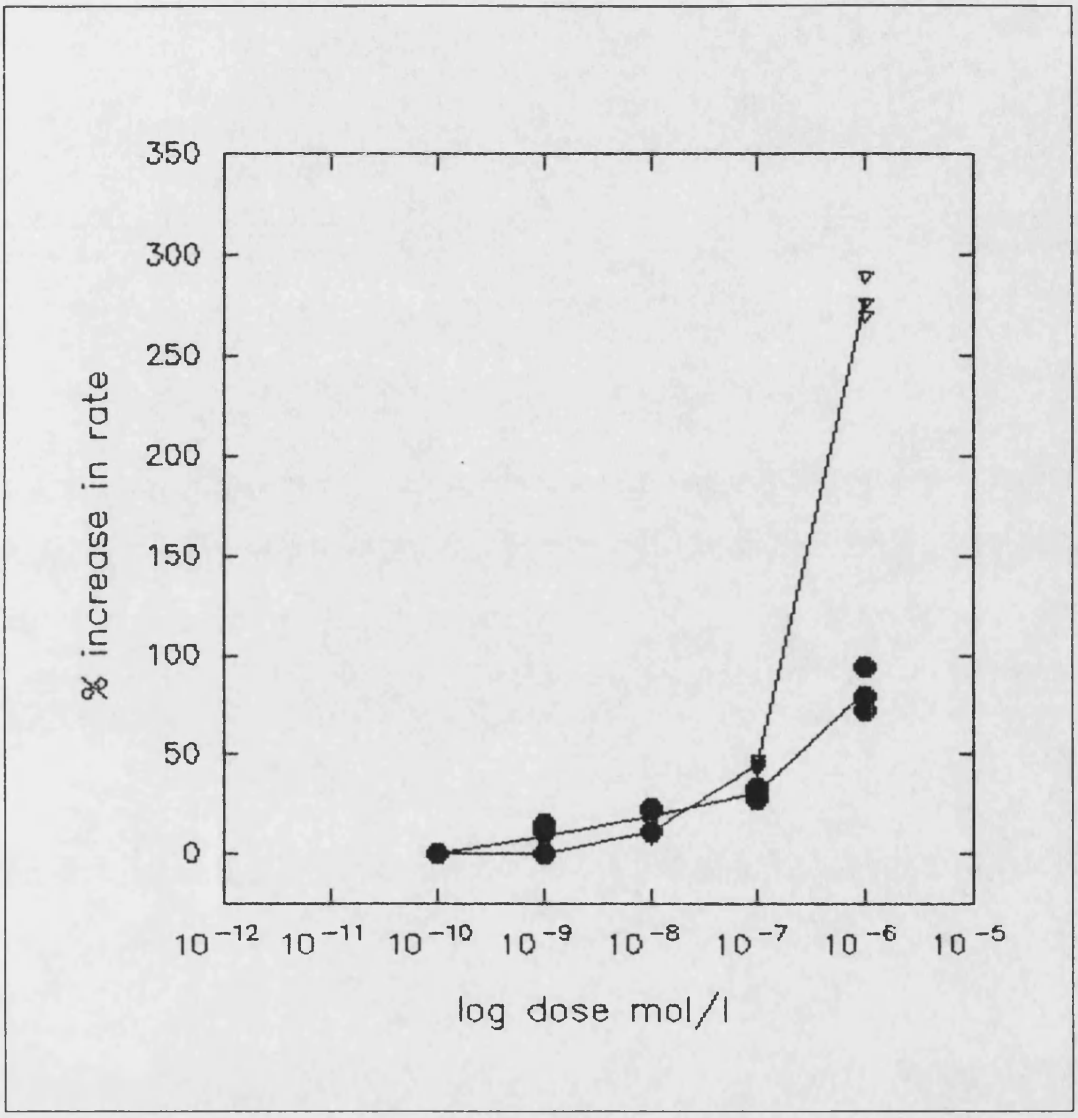

Figure 2.9: Graph showing the response of the adult heart to schistoFLRFamide and manducaFLRFamide.
SchistoFLRFamide –•– *and manducaFLRFamide* –▽–. *Conditions identical to those of the adult heart response to CCAP.*

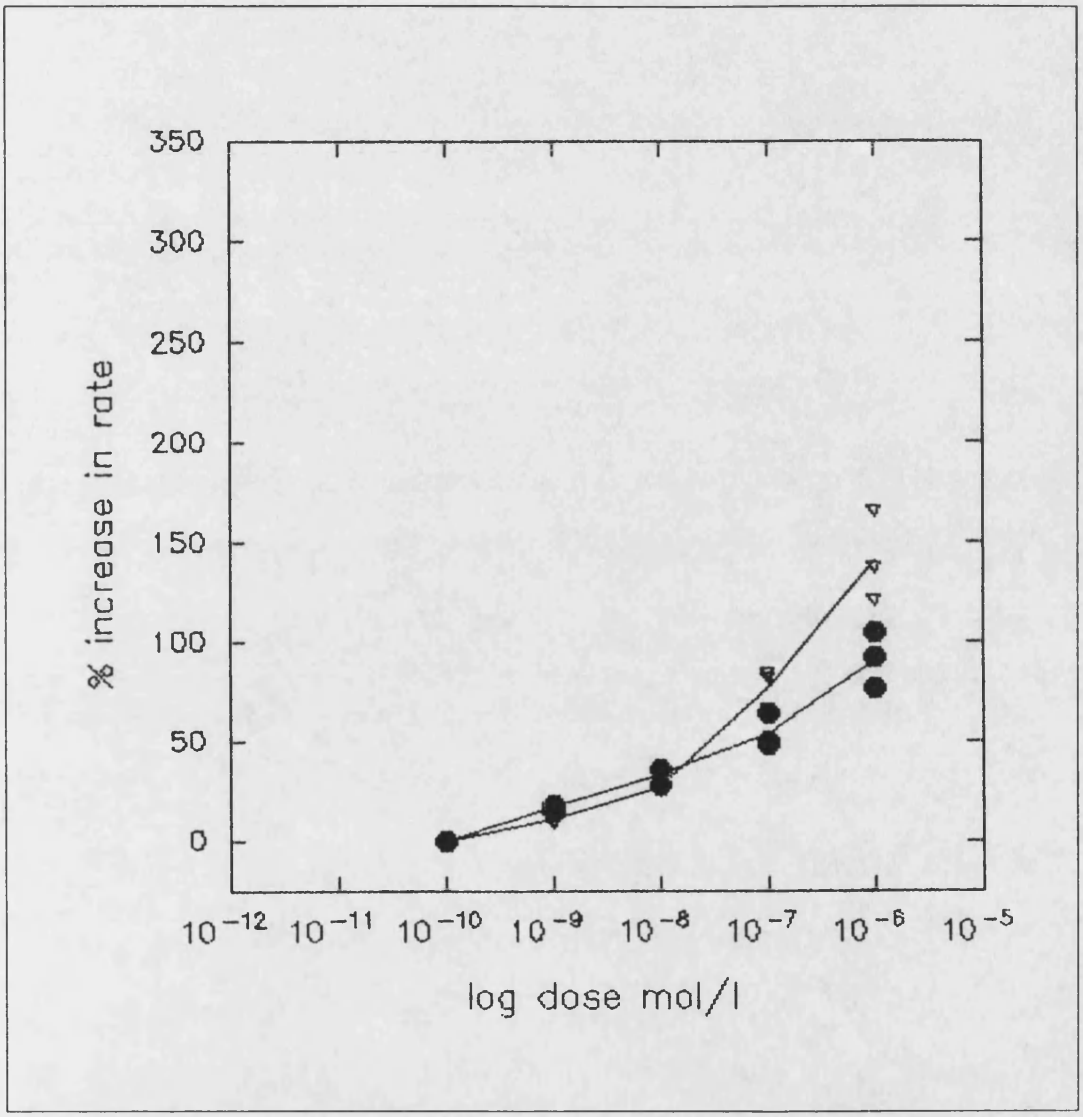

Figure 2.10: Graph showing the response of the larval heart to schistoFLRFamide and manducaFLRFamide.
SchistoFLRFamide $-\bullet-$ *and manducaFLRFamide* $-\triangledown-$. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

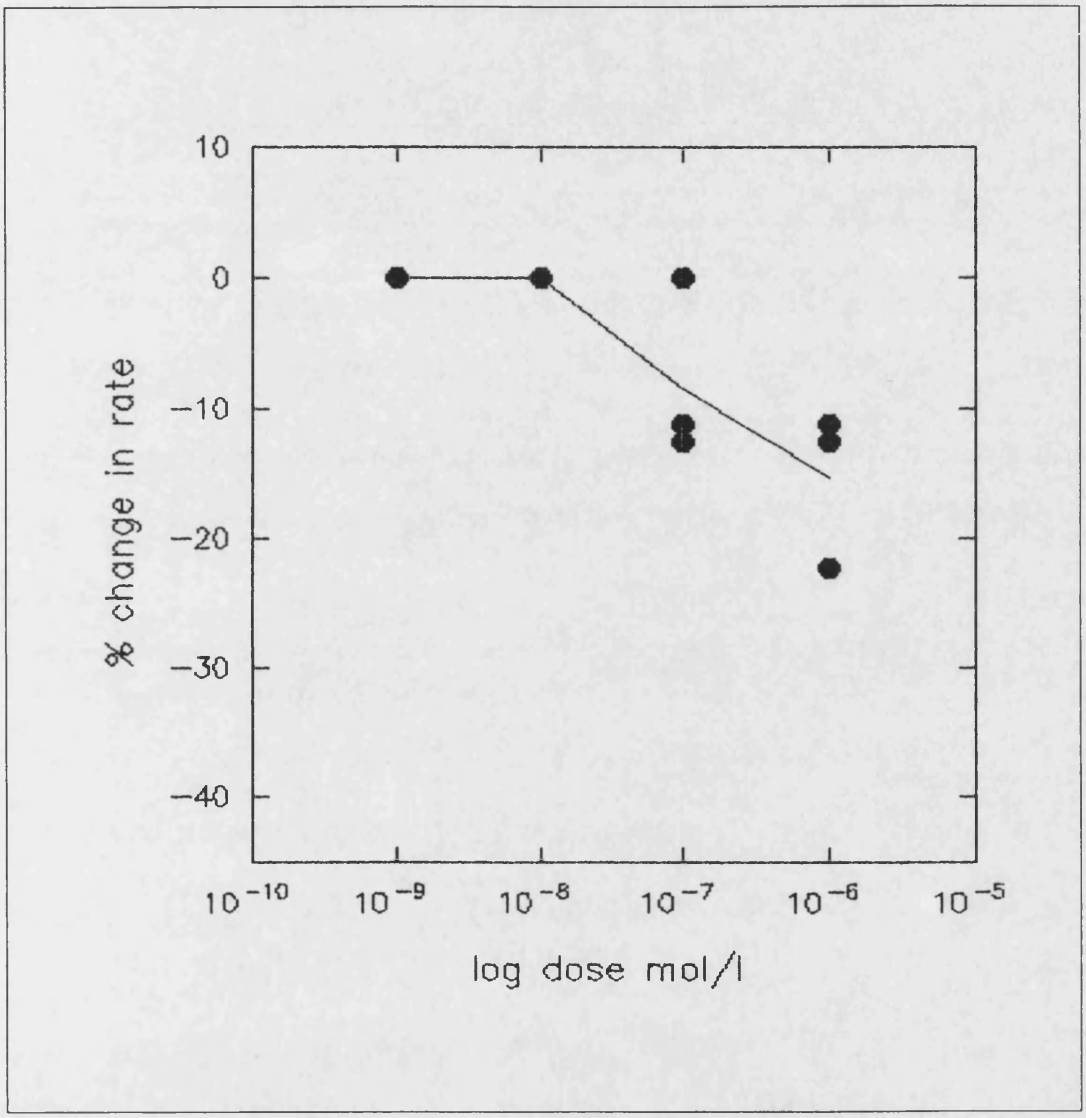

Figure 2.11: Graph showing the response of the larval heart to leucomyosuppressin. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

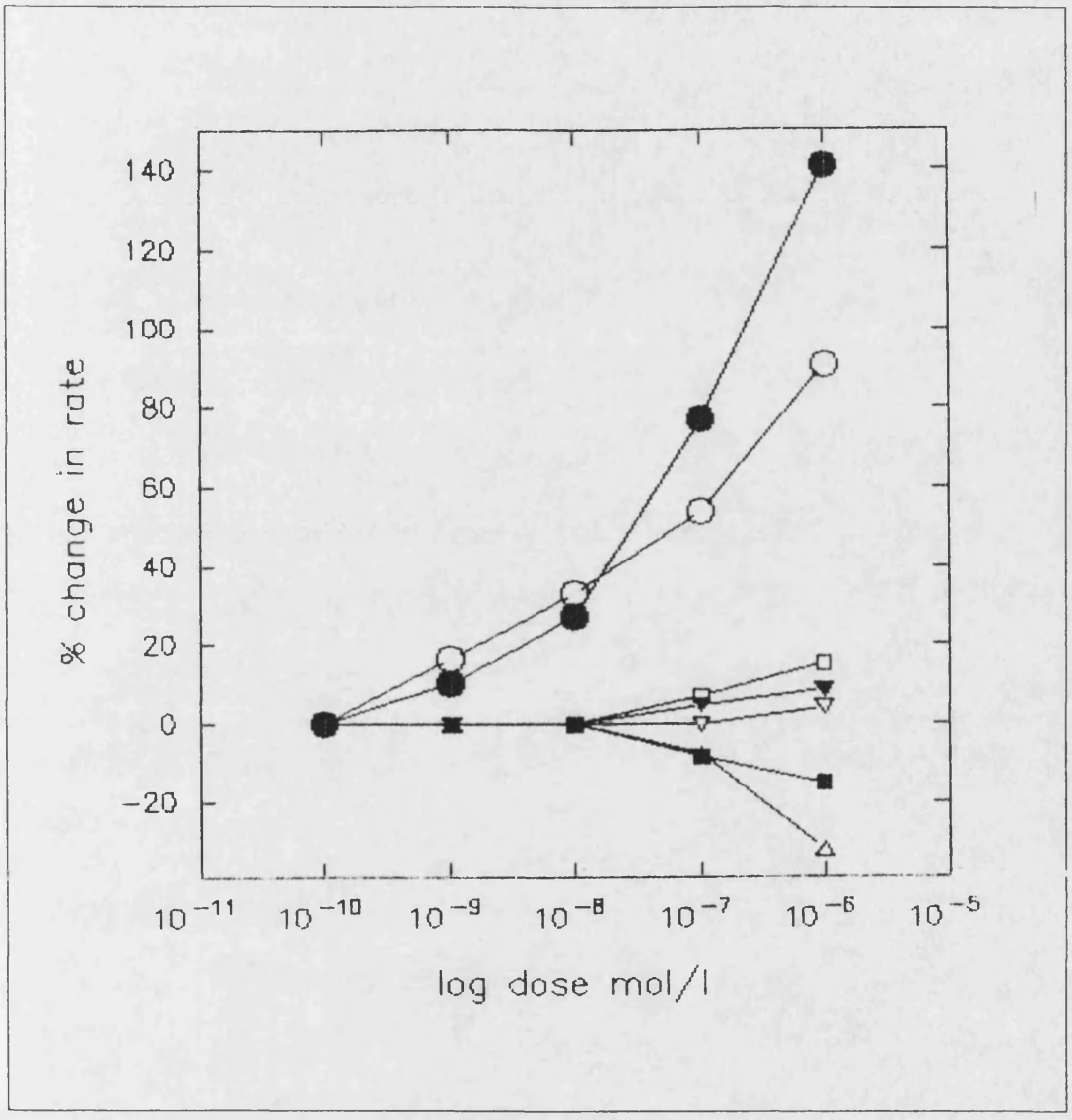

Figure 2.12: Graph showing the responses of the larval heart to extended FLR-Famide/FMRFamide peptides.

Points represent mean of three responses to schistoFLRFamide –o– and manducaFLR-Famide –•– and the response to one application of the calliFMRFamides 1 –▽–, 3 –▼– and 5 –□–, leucomyosuppressin –■– and LPLRFamide – △–. Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.

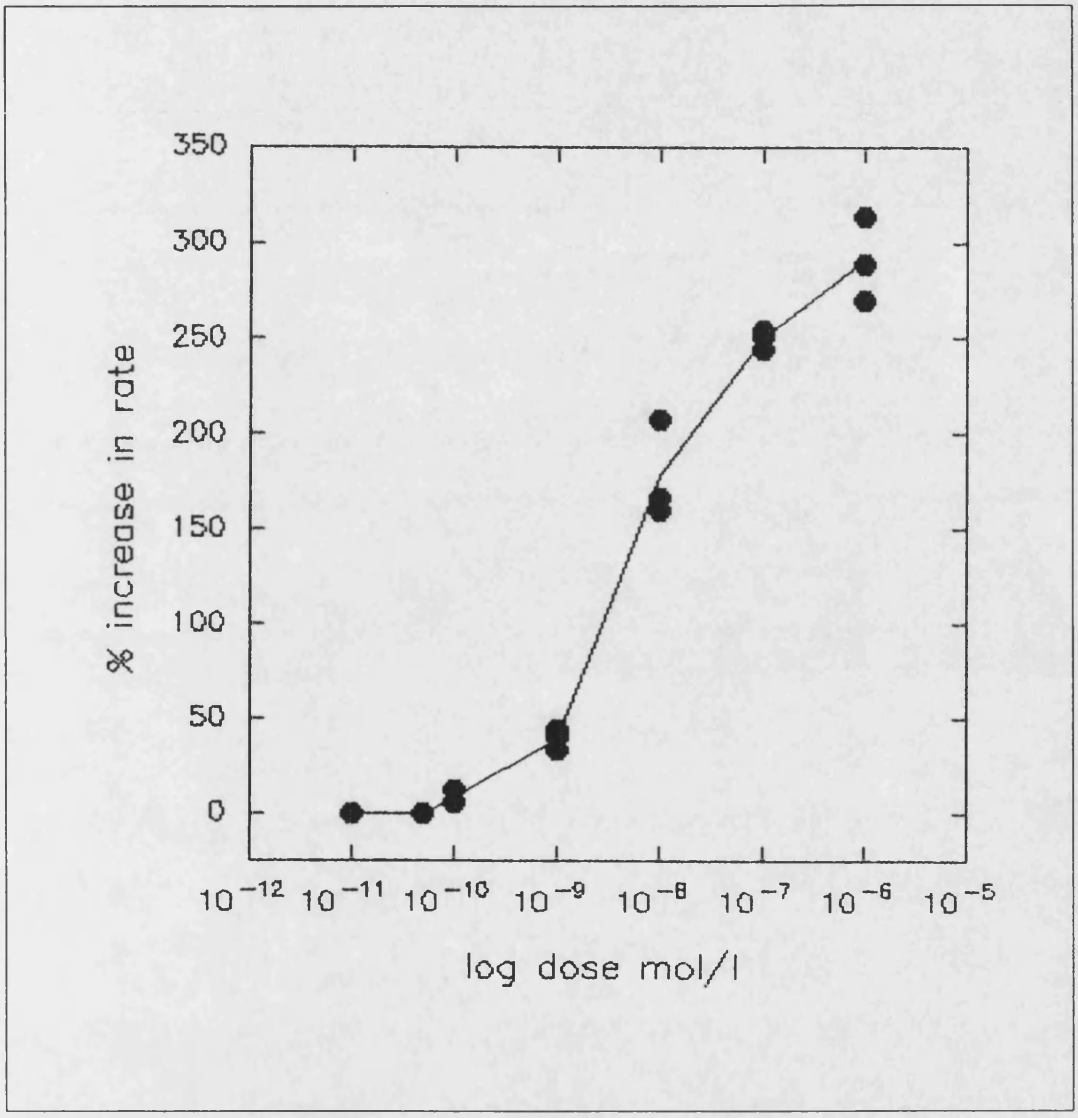

Figure 2.13: Graph showing the response of the adult heart to corazonin. *Conditions identical to those of the adult heart response to CCAP.*

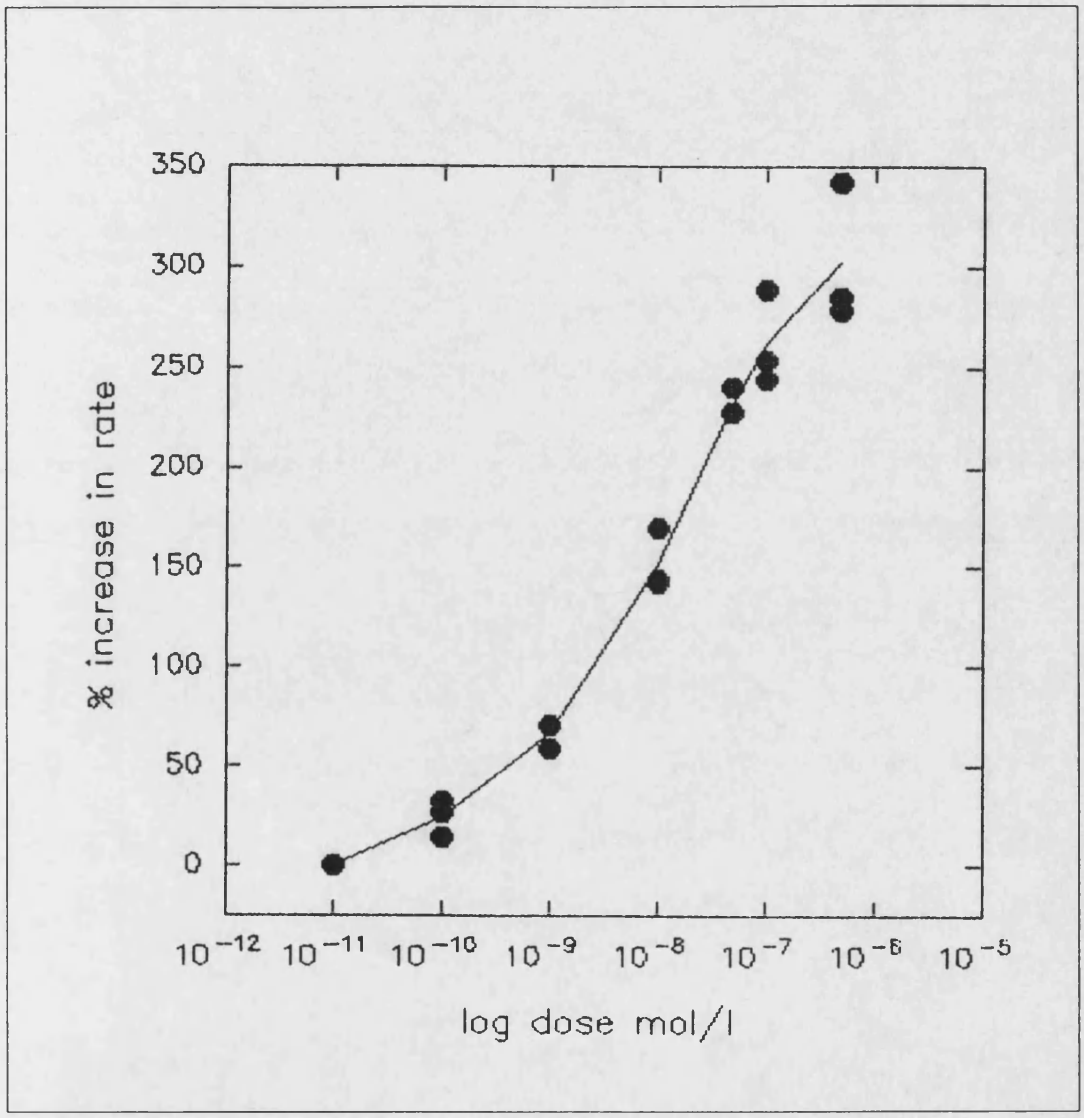

Figure 2.14: Graph showing the response of the larval heart to corazonin. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

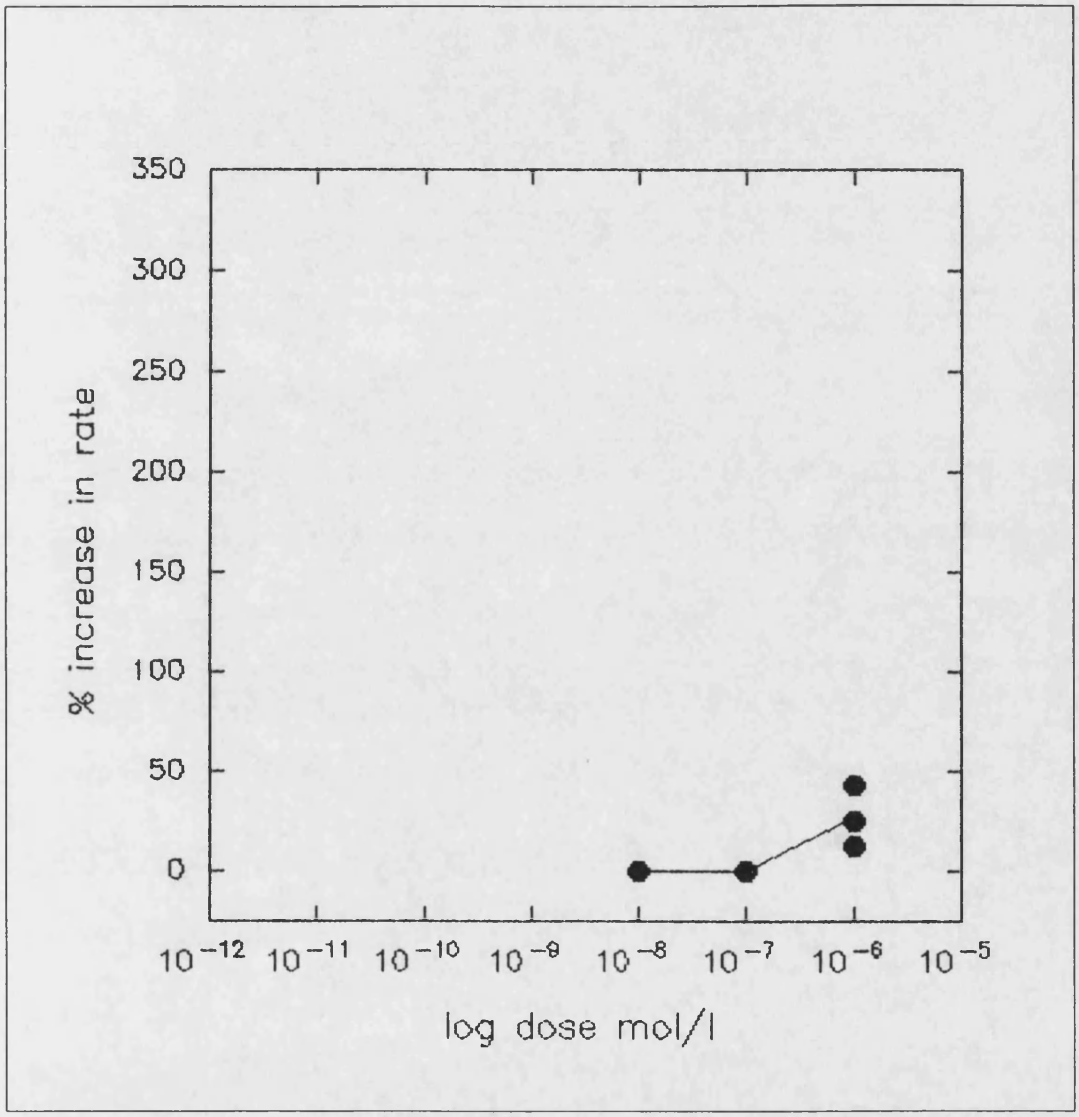

Figure 2.15: Graph showing the response of the larval heart to *Heliothis zea*-pheromone biosynthesis activating neuropeptide.
Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.

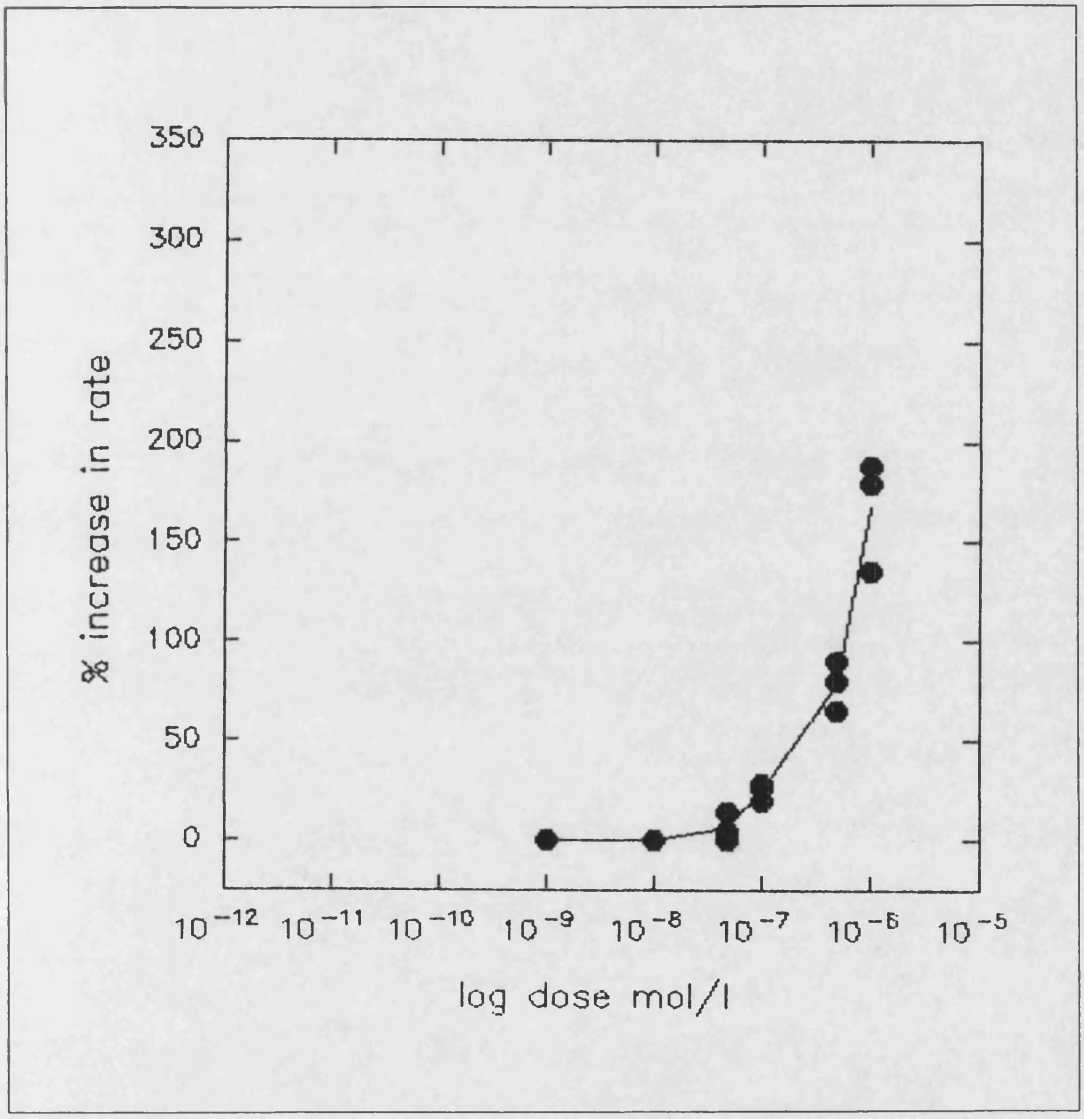

Figure 2.16: Graph showing the response of the larval heart to leucopyrokinin. *Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.*

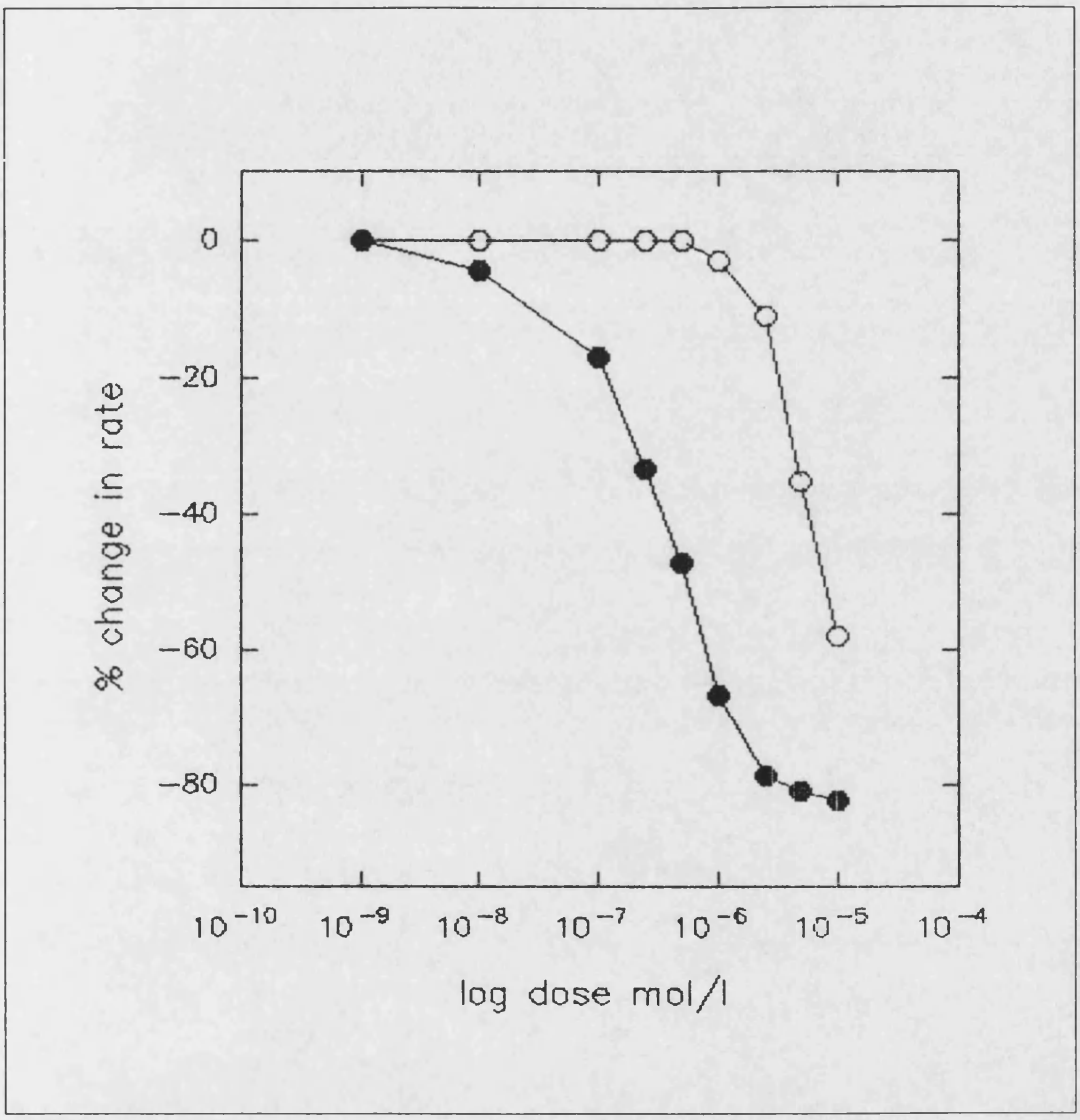

Figure 2.17: Graph showing the response of the larval heart to two of the callatostatins.

Callatostatin 1 –o– and callatostatin 5 – •–. Conditions identical to those of the adult heart response to CCAP except applications made in larval saline.

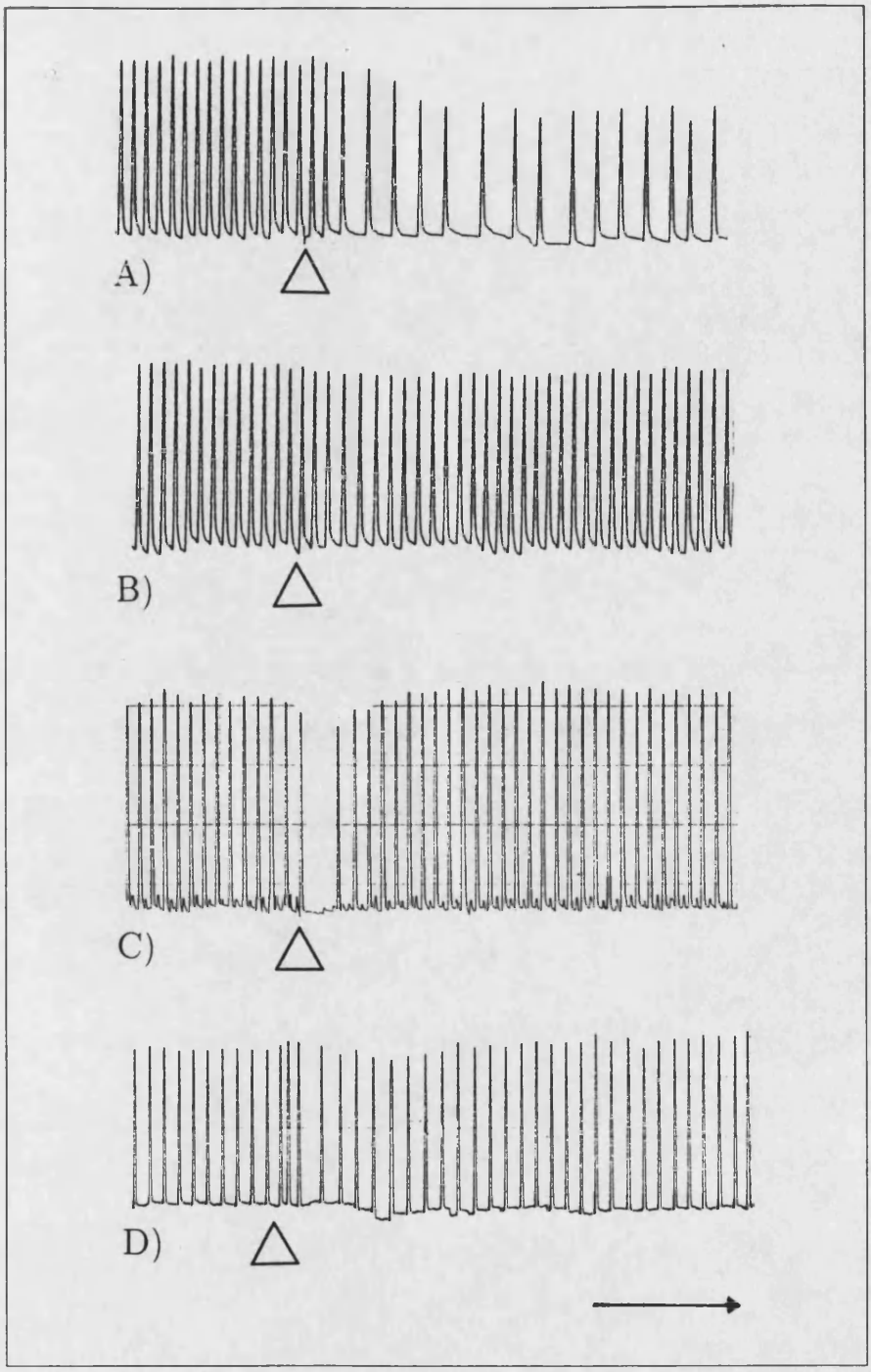

Figure 2.18: Response of the larval heart to the four inhibitory neuropeptides tested in this study.
A) callatostatin 5 – 5 x 10^{-7}M, B) callatostatin 1 – 10^{-6}M, C) LPLRFamide – 10^{-6}M and D) leucomyosuppressin – 10^{-6}M. Conditions identical to those of the adult heart response to CCAP except applications made in larval saline. A) and B) and C) and D) are from the same preparations. △ is the point of application. Horizontal arrow shows the direction of paper through the chart recorder.

2.4 Discussion

The results presented here show the responses of the *Manduca* heart to a range of insect neuropeptides. A number of the peptides tested in this study have previously been isolated from *Manduca* and are therefore known to be endogenous in this insect. Some of the other peptides used are potentially either identical or structurally similar to peptides which will possibly be identified and isolated from *Manduca* in the future.

Crustacean Cardioactive Peptide

From this range of known insect neuropeptides CCAP was prominent in having both the lowest threshold dose and in being the most cardioactive peptide in both the adult and the larval hearts. Maximum calculable response prior to systolic arrest was reached at doses of 10^{-9} M (20 fmol) in the larva and at 10^{-8} M in the adult. CCAP is clearly the most potent cardiostimulatory peptide in the range of peptides tested in this survey. Doses as low as 5×10^{-13}M (0.01 fmol) have been detected by the larval heart during the course of this investigation.

The peptide CCAP was first discovered as a cardioacceleratory substance from the pericardial organs of the shore crab (*Carcinus maenas*) (Stangier *et al.* 1987). It is a nonapeptide with an amidated C- terminus and a disulphide bridge and has no structural homology with any other known peptide. Since first being identified in the crab this peptide has been found in a range of invertebrates and appears to be a highly conserved molecule in these animals. In crustacea, authentic CCAP or CCAP immunoreactivity (CCAPIr) has been detected in the crabs *Carcinus maenas* and *Cancer pagurus* (Stangier 1991), the crayfish *Orconectes limosus*

(Stangier and Keller 1990), *Astacus leptodactylus* and *Astacus astacus* (Audhem *et al.* 1993), and the lobster *Homarus americanus* (Dircksen 1994), and also in lower crustaceans (review: Dircksen 1994). From among insects, CCAP has been isolated from *Locusta migratoria* (Stangier *et al.* 1989), *Manduca sexta* (Hildebrand *et al.* 1990, Cheung *et al.* 1992, Lehman *et al.* 1993), *Spodoptera eridania* and *Tenebrio molitor* (Furuya *et al.* 1993). In addition, CCAPIr has been found in *Periplaneta americana* (Davies *et al.* 1990), *Gryllus bimaculatus* (Sporhase-Eichmann *et al.* 1991) and *Calliphora vicina* (Jahn *et al.* 1991). The occurrence of CCAP in a number of phylogenetically divergent groups of arthropods and the conservation of the primary structure of the peptide point to it being an important factor in the physiology of this group of animals.

Evidence as to the role of CCAP in the neurophysiology of arthropods is not extensive. The heart appears to be a target organ in all arthropods which have been studied with CCAP acting as a cardioacceleratory neurohormone. In *Carcinus maenas* the peptide is predominantly concentrated in the neurohaemal pericardial organs which would suggest a neurohormonal function. Release is triggered in high K^+ saline by a Ca^2+ dependent release mechanism (Stangier *et al.* 1988). The retinal cells and stomatogastric ganglia (Weimann *et al.* 1992) are also likely to be targets for this peptide. In insects, CCAP is cardioactive in *Locusta* (Dircksen *et al.* 1991), in *Manduca* (Davies *et al.* 1990, Loi *et al.* 1992, Cheung *et al.* 1992 and this study) and in the blowfly *Calliphora vicina* (Jahn *et al.* 1991). Work with locusts has established that, in addition to the heart, putative targets of this peptide include visceral muscles such the oviducts (Burdzik 1990) and hindgut (Stangier *et al.* 1989) and some skeletal muscles (Lehman *et al.* 1993).

Immunohistochemical studies (Davies *et al.* 1993) of CCAP distribution in the abdominal nerve cord of *Manduca* have shown that the peptide is produced in a

set of neurosecretory cells which terminate in the lateral nerves (see also Chapter IV of this study). These nerves have been shown to be the main site of neuropeptide release from the nerve cord in the insect (Raabe 1982), therefore it is likely that CCAP is released from neurohaemal organs located on the lateral nerves and circulates via the haemolymph to the heart tissues. There is no clear evidence that the heart in *Manduca* is directly innervated by neurones containing CCAP but CCAPIr neurones extend from the transverse nerve via a peripheral neurosecretory cell (NS-L1) to the alary muscles (see Fig. 1.3) (Davies *et al.* 1993).

In *Manduca*, CCAP has been found to be identical to CAP2a. This peptide is one of the three CAP2s isolated from *Manduca* of which only this sequence is known (Loi *et al.* 1992). Previous extensive work on the CAP2 peptides has identified a probable role for these peptides during specific stages in development; absorption of the external yolk sac in the embryo (Broadie *et al.* 1990), purging of the gut prior to pupation (Broadie *et al.* 1989, Tublitz *et al.* 1992), at wing inflation after emergence from the pupal case and during flight episodes (Tublitz 1989).

The results from the present study support the evidence that CCAP is an important cardiostimulatory neuropeptide in *Manduca sexta.* The steep dose response curves and the low threshold doses required to obtain a maximum observable response point to receptors specific to CCAP being present in the myocardium.

Both the adult and the larval hearts are highly sensitive to the peptide with the results suggesting that the larval heart may be more responsive. This may reflect the differences in the two bioassay techniques and the structure of the hearts. The delicate, more open structure of the larval heart means that samples applied in a pulse as in these experiments may reach receptors in the myocardium more

effectively than in the more tube-like adult heart. Evidence from this and previous studies (Platt 1984) has however demonstrated that the adult heart can be more responsive to lower doses of cardioactive factors than the larval suggesting that the differences in threshold doses are probably actual rather than artefactual.

The greater sensitivity of the larval heart to CCAP seen in this study does not fit in with the putative roles of the CAP2 neuropeptides in the adult and larval stages. The probable target tissue of the CAP2s in the larva has been identified as the gut (Broadie *et al.* 1989, Tublitz *et al.* 1992) whilst in the adult the heart is a more likely target (Tublitz 1989). The results from this study suggest that the larval heart is highly responsive to this peptide and that the *Manduca* heart loses some responsiveness to CCAP during metamorphosis.

As CCAP is now known to be one of a family of cardioactive neuropeptides, it would be reasonable to assume that these peptides could be released independently from one another and that not all of the peptides perform all of the functions attributed to the CAP2s. One explanation for the apparent change in responsiveness is that in the larva CCAP may be more important as a heart regulatory peptide than for the adult in comparison to other members of the CAP2 family. The differences in sensitivity of the adult and larval hearts to members of the CAP2 family are shown in Figs. 2.4 and 2.5. When applied at equivalent concentrations, the adult heart is most responsive to CAP2c, then CAP2b and is least responsive to CAP2a (CCAP). The larval heart, in comparison is most responsive to CAP2a (CCAP), then CAP2c and is least responsive to CAP2b. The cell population identified by an antibody that recognises an epitope common to some of the CAPs (Taghert *et al.* 1983, 1984) changes during larval to adult development which may indicate a change in the neuropeptide produced by these cells (see Fig. 2.19). The evidence of the differences in responsiveness

to the CAP2s between adult and larval hearts supports the suggestion that the members of the CAP2 family act at different times during development and are likely to have different target organs in the different stages.

Manduca sexta Allatotropin (Mas-AT)

Mas-AT is a neuropeptide originally isolated and sequenced from adult *Manduca sexta* in which it stimulates the synthesis of juvenile hormone in corpora allata of adult moths (Kataoka *et al.* 1989). The sequence of this peptide has some close similarity to that of one of the locust myotropins isolated from the accessory gland of this insect, Lom-AG-myo I (Paeman *et al.* 1991a). Both the peptides have identical sequences of five amino acid residues at both the C and the N termini (see Fig. 2.3).

Immunohistological evidence of Mas-AT has been found in the brain, retrocerebral complex and abdominal nerve cords of adult moths (Veenstra and Davies 1993) but no Mas-AT immunoreactive material has been detected in the larva (Veenstra *et al.* 1994). Mas-allatotropin has no effect on the rate of juvenile hormone biosynthesis in larval or pupal corpora allata (Kataoka *et al.* 1989) but is active in the adult, suggesting that the allatotropic activity of this peptide is restricted to the adult stages.

The results from the present study show that whilst the peptide is highly cardiostimulatory in the adult heart the larval heart is unresponsive to Mas-AT even at high doses. These findings are in agreement with the distribution detected by immunohistochemical staining (Veenstra *et al.* 1994) where evidence of Mas-AT was only seen in adult moths. The allatotropin is only produced in the adult

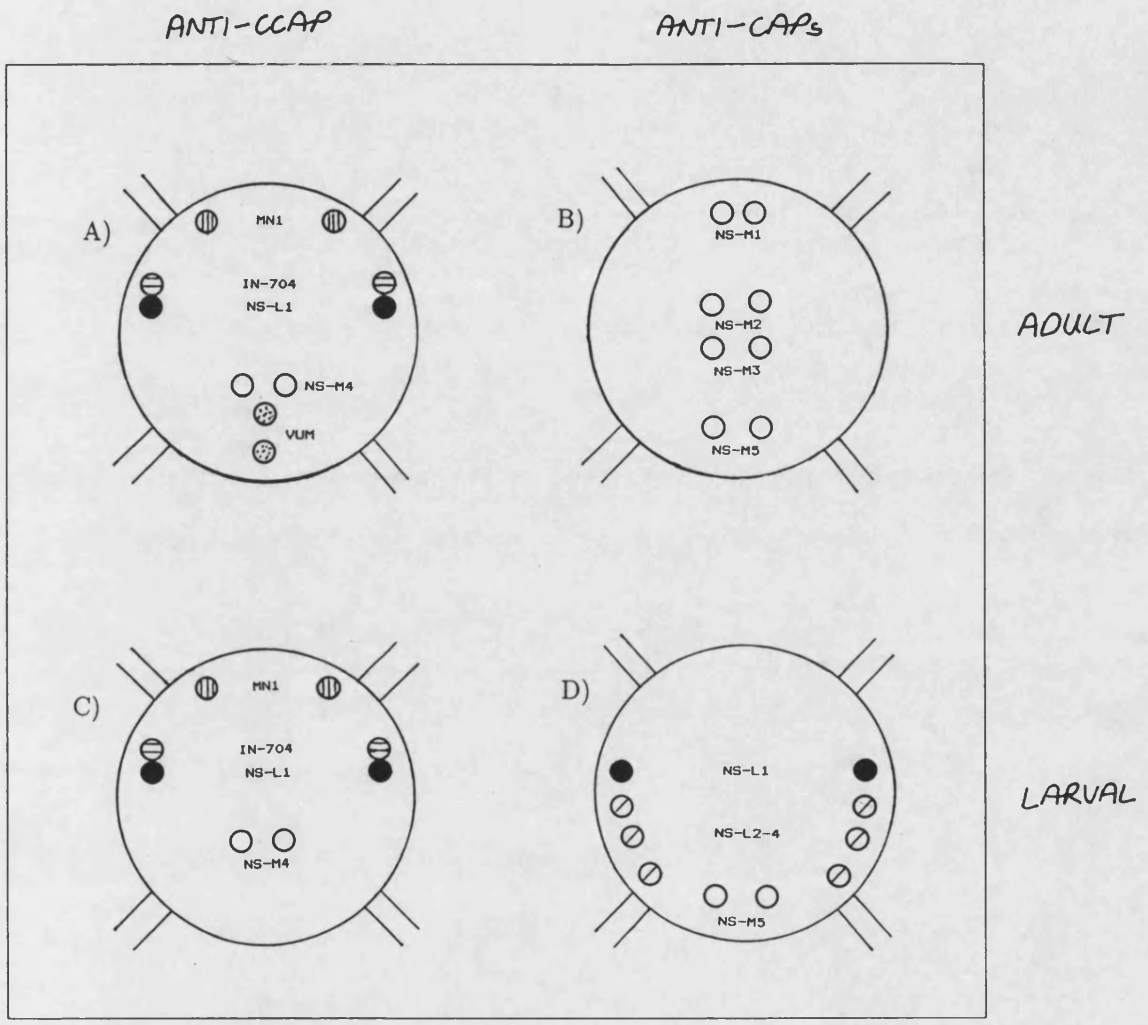

Figure 2.19: Diagrammatic representation of the immunolocalisation of cardioactive peptides in the unfused ganglia of the abdominal nerve cord of *Manduca sexta*.
A) Adult anti-CCAP neurons (Davies et al. 1993), B) Adult anti-CAP neurons (Taghert et al. 1983, 1984), C) Larval anti-CCAP neurons (Davies et al. 1993) and D) Larval anti-CAP neurons (Taghert et al. 1983, 1984).

moth and only the adult myocardium has receptors to this peptide.

Mas-AT alone of the peptides tested in this study was cardioactive in the adult and not the larval heart. This specificity of response is also seen for the CAPIs (Tublitz *et al.* 1992) which are two unidentified peptides from the abdominal nerve cords of adults (Loi *et al.* 1992). This observation led Veenstra *et al.* (1994) to speculate that Mas-AT is one of the as yet unidentified CAPIs. The evidence given for this suggestion is not extensive. Immunohistochemical investigation into the distribution of Mas-AT in the nervous system of the adult moth has shown that three pairs of midline neurosecretory cells are immunoreactive to anti-Mas-AT antiserum: NS-M1, NS-M2 and NS-M5 (Veenstra *et al.* 1994). These midline neurosecretory cells are a subset of the five pairs of midline neurosecretory cells identified by Taghert and Truman (1982). All these cells have projections to the PVOs, the neurohaemal organs of the abdominal nerve cord, and have been termed NS- M1-M5 after Davies *et al.* (1993). A monoclonal antibody which purportedly recognises epitopes common to both CAPIs and CAP2s detected immunoreactive material in NS-M1, NS-M2, NS-M3 and NS-M5 (Tublitz and Sylwester 1990) (see Fig. 2.19). Three of pairs of cells are therefore, immunoreactive to both the anti-allatotropin antiserum and the anti-CCAP antibody (NS-M1, NS-M2 and NS-M5) (Veenstra *et al.* 1994). A second antibody shown to be specific for one of the CAP2s, CCAP is only immunoreactive to NS-M4. This provides evidence that Mas-AT is either co-localised with the CAPIs in the neurosecretory neurones or is itself one of these peptides.

The CAPIs are released together with the CAP2s at the time of eclosion and later during flight (Tublitz and Truman 1985c, Tublitz and Evans 1986, Tublitz 1989). That Mas-AT is released at this time also has as yet not been shown but as the CAPIs consist of more than one peptide (Loi *et al.* 1992) it cannot be assumed

that release of all these peptides is simultaneous. Different members of this set of peptide may be released at varying times. Sequencing of the CAP1s has so far been unsuccessful. CAP1s are known to be released together with CAP2s at eclosion and during flight episodes in the adult (Tublitz 1989) but attempts to isolate Mas-AT from haemolymph at these times have also been unsuccessful (Veenstra *et al.* 1994).

In summary, the evidence that Mas-AT is a CAP1 peptide lies in the specificity of the response of the heart bioassays, immunohistochemical evidence of Mas-AT restricted to adult moths and in the immunohistochemical evidence of co-localisation of Mas-AT and the CAPs. Data presented in later chapters of this study does not wholly support the suggestion of Veenstra *et al.* (1994) as Mas-AT was found to be myoactive on the oviduct of *Manduca* whereas the CAP1s were not (see also Chapters III and IV of this study). HPLC extractions of *Manduca* nervous system (see Chapter V) show however that the CAP1s and synthetic Mas-AT elute at a similar time from a solvent gradient.

The evidence from this study showing the low threshold dose value and the steep dose response curve of the peptide in the adult heart signifies that receptors specific to this peptide occur in the tissues of the adult heart. The responses obtained suggest that Mas-AT is cardioactive in the heart of the adult *in vivo* and give additional support for a role of Mas-AT in the elevation of the heartrate during wing inflation or flight behaviour.

The question as to the identity of the CAP1s and the relationship, if any to Mas-AT is likely to remain unresolved until the CAP1s are isolated and sequenced.

Locustatachykinin II (Lom-TK II)

Tachykinins are a family of structurally related peptides originally isolated from vertebrates. Locustatachykinins (Lom-TKs) were the first members of this family to be isolated and identified from the central nervous system of invertebrates (Schoofs *et al.* 1990a,c). Since this time further members of the tachykinin family have been isolated from insects, sialokinins I and II from the mosquito *Aedes aegypti* (Champagne and Ribeiro 1994) and callitachykinins from *Calliphora vomitoria* (Lundquist *et al.* 1994).

Five Lom-TKs were isolated from *Locusta* using the *Leucophaea maderae* hindgut to detect myoactivity. All have the carboxyterminal hexapeptide Gly-Phe-Tyr-Gly-Val-Arg-NH_2 in common except Lom-TK IV which has His rather than Tyr in this sequence (see Table 2.4). Four of the Lom- TKs have Ala-Pro at the N terminus. For the purposes of this study Lom-TK II was selected to best represent the locustatachykinin family as this member has both the features carried by most of the other members.

Locustatachykinins stimulate the contractions of the hindgut in *Leucophaea maderae* (Holman *et al.* 1991) and of the oviduct and the foregut in *Locusta migratoria* (Schoofs *et al.* 1990a,c). In the moth *Bombyx mori* locustatachykinins stimulate pheromontropic activity in the pheromone glands (Fonagay *et al.* 1992). Tachykinin-related peptides have also been isolated from the blowfly *Calliphora vomitoria* (Lundquist *et al.* 1994). These results would suggest that tachykinin-like peptides are widespread among insects.

As yet no member of the tachykinin family has been detected in *Manduca* however previous work (Reynolds, unpublished observations) showed that Lom-TK I and

II are potent cardiostimulators of the larval heart of *Manduca*. The results from this study have confirmed that whilst Lom-TK II is approximately 10 fold less cardiostimulatory than CCAP, the peptide is nevertheless strongly excitatory in the larval heart. From these results it would be reasonable to assume that tachykinin-like peptide receptors are present in the myocardium of the larval *Manduca* and therefore that tachykinin-like neuropeptides are to be found in the larval stages of this insect.

Two cardioactive factors termed CAF1 and CAF2 have been partially isolated from extracts of the abdominal nerve cord of the larval *Manduca* (Platt and Reynolds 1985). The CAF2 fraction has since been shown to be identical to the CAP2 group from adult moths (Tublitz *et al.* 1992), a set of three peptides of which one is CCAP (Cheung *et al.* 1992). The CAF1 fraction was shown to be cardioactive only in the larva and has yet to be identified. The data presented in this study and the detection of anti-Lom-TK II immunostaining in the ganglia of the larval nerve cord (A. K. Marshall, unpublished observations) indicates that the larval stages of this insect possess tachykinin-like peptides. The specificity of the myoactivity to the larval heart of these peptides and the likelihood of their presence in the larva suggests that CAF1 may contain at least one tachykinin-related peptide.

Confirmation of the presence of tachykinin-like neuropeptides in *Manduca* larvae must await further work towards the isolation of myoactive factors from the nervous system of this stage of the insect.

FLRFamide-related Peptides

The neuropeptide FMRFamide was first isolated and sequenced from extracts of the nervous system of the clam *Macrocallista nimbosa* (Price and Greenberg 1977). Since the discovery of FMRFamide, a number of structural homologues all of 4-18 amino acids in length and having a C terminus ending in Arg-Phe-amide have been identified from a wide range of phyla. These peptides have been shown to have a diverse range of activities in both vertebrates (Roth *et al.* 1987, Yang *et al.* 1985, Raffa 1988) and invertebrates (Li and Calabrese 1987, Price *et al.* 1987, Lehmen and Greenberg 1987).

FMRFamide is usually regarded as being the root member of this interphyletic family of structurally related peptides however a large subset of the family is related instead to the peptide FLRFamide (reviews: Greenberg *et al.* 1988; Price and Greenberg 1989; Walker 1992).

Studies of FLRFamide-like peptides in insects have investigated the modulation of muscle activity in both somatic and visceral by these peptides; in the heart (Cuthbert and Evans 1989, Duve *et al.* 1993), in the oviduct (Peeff *et al.* 1993), in the hindgut (Holman *et al.* 1986) and in the leg skeletal muscle (Evans and Myers 1986, Elia and Orchard 1995). FLRFamide-like peptides can induce contraction and stimulate or inhibit spontaneous activity.

In insects FLRFamide-like peptides have been isolated from the locust *Schistocerca gregaria* (Robb *et al.* 1989), *Manduca sexta* (Kingan *et al.* 1990, Witten *et al.* 1993), the blowfly *Calliphora vomitoria* (Duve *et al.* 1992), the cockroach *Leucophaea maderae* (Holman *et al.* 1986), the fleshfly *Neobelliaria bullata* (Fonagy *et al.* 1992b) and the fruit fly, *Drosophila melanogaster* (Nichols 1992) (see

Table 2.5). The number of species from which these peptides have been isolated or in which they have been immunologically detected points to a widespread distribution among insects.

SchistoFLRFamide is a potent cardioinhibitor of the heart of *Schistocerca gregaria* with a threshold of between 10^{-9} and 10^{-10}M (Robb *et al.* 1989). An identical peptide has been isolated from *Locusta migratoria* (Schoofs *et al.* 1993a) based on its ability to suppress the spontaneous activity of the hindgut of *Leucophaea maderae*. In the locust extensor-tibiae muscle schistoFLRFamide both potentiates and inhibits the twitch response (Robb and Evans 1990) and is inhibitory in the oviduct of *Locusta migratoria* (Schoofs *et al.* 1993a). SchistoFLRFamide-like immunoreactivity in *Locusta* is present in the corpora cardiaca, the neurohaemal organs of the abdominal nerve cord, the salivary glands, the muscles of the oviduct and heart, and in the male accessory glands (Schoofs *et al.* 1993a). The distribution suggests that these peptides play both a neurotransmitter and a neurohormonal role, acting both locally at nerve terminals and in the circulation.

The peptide manducaFLRFamide was first isolated from brain sub-oesophageal ganglia extracts of adult moths (Kingan *et al.* 1990) using an ELISA. Immunocytological evidence of manducaFLRFamide has shown that the peptide is widely dispersed throughout the CNS suggesting that it acts as a chemical messenger. Kingan *et al.* (1990) concluded that manducaFLRFamide was involved in the promotion or maintenance of flight based on the observation that the peptide increases the force of neurally invoked contractions in an indirect flight muscle, the dorsal longitudinal. ManducaFLRFamide suppresses the spontaneous contractions of the midgut of the sphingid moth, *Agrius convolvuli* at low doses and immunolocalisation of the peptide adds supportive evidence for its activity in the regulation of the digestive tract (Fujisawa *et al.* 1993).

The calliFMRFamides were isolated from *Calliphora vomitoria* (Duve *et al.* 1992) with the use of radioimmunoassay and HPLC. Some of these peptides were found to be active in inducing fluid secretion from the salivary gland. Six structurally similar calliFMRFamides were applied to the semi-isolated blowfly heart, together with schistoFLRFamide and leucomyosuppressin (Lem-MS). Despite close sequence similarities the peptides varied greatly in their ability to modulate the heart. Evidence from these structure–activity studies indicates that both the C and the N-terminal regions of these peptides are important for their cardioactivity (Duve *et al.* 1993). The N-terminal allows the recognition of the peptides by the receptor and confers specificity on the interaction whereas the C-terminal is crucial for activity.

Lem-MS from the head of the cockroach *Leucophaea maderae* is a potent inhibitor of the spontaneous contractions of the foregut and hindgut of this insect (Holman *et al.* 1986d). In the blowfly, Lem-MS inhibits the heart at high doses (10^{-6} M, Duve *et al.* 1993) and is also inhibitory in the oviduct of *Locusta migratoria* (10^{-7} M, Peeff *et al.* 1993). SchistoFLRFamide shows a high degree of homology with Lem-MS. The two peptides are identical except for the N-terminal amino acid of proline in schistoFLRFamide and pyroglutamate in Lem-MS. MasFLRFamide differs from Lem-MS by two residues but has a pyroglutamate at the N-terminal in common. Despite considerable sequence homology, these peptides have do not consistently display the same activities. In the blowfly heart, schistoFLRFamide is inactive (Duve *et al.* 1993) whereas Lem-MS is inhibitory, but in the locust oviduct, the two peptides have similar activities (Peeff *et al.* 1993).

The results from the present study demonstrate that none of the members of the FLRFamide family tested are very active on the heart of *Manduca sexta* in comparison to other cardiostimulatory peptides. The indication from this study

is that the heart is probably not the target organ for these peptides in this insect.

In the larval heart of *Manduca sexta*, both schistoFLRFamide and the native FLRFamide found in *Manduca*, Mas-FLRFamide, were cardiostimulatory. Mas-FLRFamide although being a marginally more potent cardiostimulator in high doses has a similar dose response curve to schistoFLRFamide. This would appear to suggest that the N-terminal sequence is not of primary importance to receptor binding as Mas-FLRFamide has an pGlu and schistoFLRFamide has an Pro at this position. Lem-MS is inhibitory in this bioassay as is the peptide LPLR-Famide. These peptides have no similarity other than the LRFamide C-terminal sequence.

The peptide LPLRFamide was first extracted from chicken heads (Dockray *et al.* 1983) and has not been found in any insect species. This peptide is slightly more cardioinhibitory than Lem-MS in the larval heart but in addition, shows a different response profile with rapid cessation of the heartbeat and quick recovery rather than a biphasic response of initial acceleration followed by inhibition and slow recovery (shown in Fig. 2.18). The other peptides found to be cardioinhibitory in the *Manduca* heart, the callatostatins 1 and 5, also gave a different response with slow reduction in rate followed by slow recovery.

The calliFMRFamides were tested only on the larval heart and showed an order of magnitude less cardioactivity from that of either schistoFLRFamide or Mas-FLRFamide in this preparation. This together with the evidence that the peptide FMRFamide is inactive on the *Manduca* heart possibly points to the importance of the N-terminal sequence being either pGlu-Asp-Val or Pro-Asp-Val for cardioactivity or alternatively the importance of the carboxy terminal sequence Phe-Leu-Arg-Phe- NH_2 rather than Phe-Met-Arg-Phe-NH_2.

The results from this study strongly suggest that more than one receptor is present in the larval heart for FLRFamide- related peptides with one receptor causing inhibition of contraction and another stimulation. In the locust oviduct, inhibition is determined by C-terminal amidation and a specific N-terminal extension (Peeff *et al.* 1993). In the *Manduca* heart, both the amino acid sequence and also the length of the molecule appear to be of importance in receptor specificity. The LRFamide sequence allows receptor recognition and the sequence of the N-terminal extension determines stimulatory or inhibitory activity. The N terminal sequence of pGlu-Asp-Val- seen in both Mas-FLRFamide and Lem-MS does not however, determine activity. The two cardiosuppressive peptides from this study are both of eight amino acid residues or less. This property and the possession of an N-terminal extension of the root LRFamide sequence could be a requirement for cardioinhibition. Alternatively or in addition, small differences in the primary structures alter the conformation of the peptide and could therefore confer variation in physiological effects. Further study of the structure–activity relationships of this group of peptides would be required before the determinants of activity could be deduced.

Corazonin

Corazonin was first isolated and characterised from the American cockroach *Periplaneta americana* (Veenstra 1989). Corazonin has subsequently been isolated from the cockroach *Nauphoeta cinerea* and characterised in *Manduca* and *Schistocerca gregaria* (Veenstra 1991). The sequence in the locust differs by one residue from the other species in having a histidine at position 7 rather than arginine however the peptide appears to occur generally in insects and be well conserved. Corazonin has structural and functional similarities to members of the

AKH/RPCH (adipokinetic hormone red pigment concentrating hormone) family, some members also stimulate heartrate but the family is diverse and appears to have undergone rapid evolution.

The fact that corazonin is blocked at both ends of the molecule suggests that it is of physiological significance. In the cockroach it is known to be cardioactive at physiological concentrations (Veenstra 1989). Predel *et al.* (1994) and Veenstra and Davies (1993) notes that in both *Periplaneta americana* and *Leucophaea maderae*, corazonin is sythesised in neurosecretory cells in the brain whose axons terminate in the corpus cardiacum. The peptide is present in sufficient quantity to be a factor in the control of the heartbeat of this insect (Veenstra 1991, unpublished results).

The distribution of the peptide in the *Manduca* nervous system has not been elucidated, however it is not wholly unreasonable to suppose that this is similar to that of *Periplaneta.* The results obtained in this study show that corazonin has a low threshold concentration in both the adult and the larval heart, suggesting that the peptide has a role in the regulation of the heart throughout the life of this insect.

Corazonin has some limited structural similarity to leucopyrokinin (Lem-PK) (Holman *et al.* 1986) which also has a pyroglutamate at the N- terminal followed by a threonine (see Table 2.6). The carboxy terminals do not show any homology. The significance of the slight similarity is uncertain. In the *Leucophaea* hindgut bioassay the bioactivity of Lem-PK remained relatively uncompromised by amino acid substitution in the N-terminal but was stongly affected by alterations in the carboxy terminal (Nachman *et al.* 1986). This signifies that in this bioassay system the active core of the peptide is in the C-terminal. The results from the

present study indicate that the cardioactivity of corazonin in the *Manduca* heart is unlikely to be mediated by the receptors that respond to Lem-PK as Lem-PK is considerably less active than corazonin in the larva and is inactive in the adult.

Leucopyrokinin (Lem-PK) and Hez-PBAN

Leucopyrokinin was isolated and sequenced from the cockroach *Leucophaea maderae* using the *Leucophaea* isolated hindgut bioassay (Holman *et al.* 1986). This peptide was the first member of a family of structurally related peptides to be identified. All the members of the family share a carboxyterminal sequence of Phe-X-Pro-Arg-Leu-NH_2 where X is Ser, Thr, Gly or Val. Included in this family are the locustamyotropins I-IV and the locustapyrokinins I-II (Schoofs *et al.* 1990d,e, 1991a, 1992a). Peptides from this family occuring in Lepidoptera include the biosynthesis activating neuropeptides from *Bombyx mori* (Kitamura *et al.* 1989, 1990) and *Heliothis zea* (Raina *et al.* 1989) and the identical melanisation and reddish colour hormone from *Pseudaletia separata* (Matsumoto *et al.* 1992), and the diapause hormone from *Bombyx mori* (Imai *et al.* 1991) (see Table 2.7). The possession of this core carboxyterminal pentapeptide sequence appears to be widespread amongst insect species.

All the members of this peptide family stimulate the hindgut in *Leucophaea maderae* and the oviduct in *Locusta migratoria* (Schoofs *et al.* 1990d,e, 1991a, 1992a). In addition, cross-functional studies have revealed that locustamyotropins stimulate pheromone biosynthesis in *Spodoptera litura* and *Bombyx mori* (Fonagy *et al.* 1992a) and cuticular melanisation in *Pseudaletia separata* (Matsumoto *et al.* 1993). PBAN stimulates visceral muscle contraction in *Leucophaea maderae* and *Locusta migratoria.* Little is known of any other physiological properties of

this family. No member of this family has as yet been isolated from *Manduca* and data regarding the cardioactive properties of these peptides in both *Manduca* and other species is lacking.

The results from this study show that both Lem-PK and Hez-PBAN are only weakly cardioactive in the larval heart of *Manduca* and not at all in the adult heart. This result would suggest that this family of peptides is not important in regulating the heart of this insect. The weak cardioacceleratory properties in the larval heart are likely to be due to cross-reactivity at the receptors in the myocardium. In the case of Lem-PK cross-reactivity may be possible with corazonin receptors as both molecules have pGlu-Thr at the N-terminal. These results do not preclude the possibility that other members of this family may be more cardioactive in *Manduca*, however this would seem unlikely because of the structural similarity of the two members tested to the rest of the family.

Proctolin

Proctolin was the first myoactive insect neuropeptide to be structurally characterised (Brown and Starratt 1975). No other structurally similar peptide is known to occur in insects. Proctolin has been identified in a wide range of arthropods and has been shown to have a diverse range of physiological properties (review: O'Shea and Adams 1986). In insects, proctolin is myoactive on a wide range of muscles including the heart of *Periplaneta americana* and *Locusta migratoria* (Miller 1979), the hindgut of *Periplaneta americana* (Starratt and Brown 1975) and the visceral muscles of the reproductive tract in *Locusta migratoria* (Lange and Orchard 1986), *Leucophaea maderea* (Holman and Cook 1985), and the horsefly *Tabanus proximus* (Cook 1981).

Proctolin has not been isolated from *Manduca* however proctolin-like immunological evidence has been noted (Davies *et al.* 1989) and in view of the widespread distribution of this peptide among arthropods it would seem unlikely not to be present in Lepidoptera.

Results obtained in this study and others (Tublitz and Truman 1980, Platt 1984) confirm that proctolin is not cardioactive in *Manduca* in either the adult or larval hearts. The distribution of proctolin immunoreactivity in *Manduca sexta* shows a paucity of proctolinergic neurons in the abdomen (Davies *et al.* 1989) and proctolin has been shown to undergo rapid enzymatic degradation in the haemolymph of *Manduca* (Quistad *et al.* 1984). This would indicate that proctolin does not act as a neurohormone in either the heart, the hindgut or the reproductive organs in this insect. The distribution of the peptide suggests a neurotransmitter role in fore and midgut activity.

Callatostatins

The term 'allatostatin' was first used to describe a group of neuropeptides capable of inhibiting the production of juvenile hormone by the corpus allatum in insects. To date, allatostatins have been identified in the cockroach *Diplotera punctata* (Woodhead *et al.* 1989, Pratt *et al.* 1989, 1991) and *Blattella germanica* (Belles *et al.* 1994), in *Manduca* (Kramer *et al.* 1991) and in the blowfly *Calliphora vomitoria* (Duve *et al.* 1993) and immunologically detected in *Locusta migratoria* and *Neobellieria bullata* (Veelaert *et al.* 1995). The allatostatin isolated from *Manduca* (Mas-AS) does not share any structural similarity with those from the cockroach species or the blowfly. The sequences of Mas-AS and the callatostatins are shown in Fig.2.8.

Although the callatostatins and cockroach allatostatins inhibit JH III secretion when tested on cockroaches, none of these peptides are allatostatic on the corpora allata of the blowfly. In addition, unlike in the cockroaches or the locust, in the blowfly, callatostatin immunoreactive neurons do not apparently project to the corpora allata. These findings suggest that callatostatins have a function other than JH III inhibition in the blowfly. Callatostatin immunopositive neurons directly innervate the heart, hindgut, rectum and oviduct (Duve *et al.* 1993, Duve and Thorpe 1994) and callatostatin 3 is a potent inhibitor of ileum motility, active at 10^{-16} to 10^{-13} M (Duve and Thorpe 1994). In the cockroach, immunolocalisation of allatostatin in the hindgut, midgut, stomatogastric system and endocrine cells of the gut (Reichwald *et al.* 1994), suggests that these peptides are brain-gut peptides.

The two callatostatins tested in this study are myosuppressive in the larval heart of *Manduca.* The threshold of the response was relatively high which may indicate the presence of structurally similar but not identical peptides in *Manduca.* The evidence that callatostatins are inhibitory in the heart of *Manduca* and the ileum of *Calliphora* whilst not having allatostatic properties in *Calliphora* confirm that callatostatin-like peptides playing a role in the inhibitory regulation of the heart and gut in some insects in addition to their activity in the regulation of juvenile hormone synthesis and release as seen in cockroaches.

2.4.1 Summary

In this study a range of insect neuropeptides was presented to the semi-isolated hearts of both the adult and larval *Manduca* and the results used to construct a series of dose response curves. The peptide CCAP was the most cardioactive

Mas-AT	Gly-Phe-Lys-Asn-Val-Glu-Met-Met- Thr-Ala-Arg-Gly-Phe-NH_2
Lom-AG-MT-I	Gly-Phe-Lys-Asn-Val-Ala-Leu-Ser-Thr-Ala- Arg-Gly-Phe-NH_2

Table 2.3: The sequences of *Manduca sexta* allatotropin and *Locusta migratoria* accessory gland myotropin I showing the close sequence similarity between these two peptides.

Lom-TK-I	Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2
Lom-TK-II	Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2
Lom-TK-III	Ala-Pro-Gln-Ala-Gly-Phe-Tyr-Gly-Val-Arg- NH_2
Lom-TK-IV	Ala-Pro-Ser-Leu-Gly-Phe-His-Gly-Val-Arg-NH_2

Table 2.4: Seqences of the locustatachykinin family.

in both stages. Mas-FLRFamide, schistoFLRFamide and corazonin were also cardioacceleratory in both adults and larvae. Mas-AT was only stimulatory in the adult while Lom- TK II, Hez-PBAN and Lem-PK were active only in the larva. The results suggest that representatives from several peptide families are present in *Manduca* but have not yet been identified in this insect. The results have given clues as to the family characteristics of peptide candidates to be the as yet unidentified CAPIs from adults and CAFIs from larvae.

FMRFamide	Phe-Met-Arg-Phe-NH_2
FLRFamide	Phe-Leu-Arg-Phe-NH_2
LPLRFamide	Leu-Pro-Leu-Arg-Phe-NH_2
SchistoFLRFamide	Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe- NH_2
Mas-FLRFamide	pGlu-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe- NH_2
Lem-MS	pGlu-Asp-Val-Asp-Phe-Leu-Arg-Phe-NH_2
Neb-MS	Thr-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2
CalliFMRFamides	
1.	Thr-Pro-Gln-Gln-Asp-Phe-Met-Arg-Phe-NH_2
3.	Ser-Pro-Ser-Gln-Asp-Phe-Met-Arg-Phe-NH_2
5.	Ala-Pro-Gly-Gln-Asp-Phe-Met-Arg-Phe-NH_2

Table 2.5: Seqences of the members of the FMRFamide/FLRFamide family tested on the heart of *Manduca sexta* in this study.

Corazonin	pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-NH_2
Lem-PK	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2

Table 2.6: The sequences of corazonin and the structurally most similar known neuropeptide, leucopyrokinin.

Lem-PK	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2
Hez-PBAN	Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln- Glu-Met- -Tyr-Gln-Pro-Asp-Pro-Glu-Glu-Met-Glu-Ser-Arg-Thr-Arg- Tyr- -Phe-Ser-Pro-Arg-Leu-NH_2

Table 2.7: Sequence similarities between leucopyrokinin and *Heliothis zea* pheromone biosynthesis activating neuropeptide.

Callatostatin 1	Asp-Pro-Leu-Asn-Glu-Glu-Arg-Arg-Ala-Asn-Arg-Tyr-Gly-Phe-Gly-Leu- NH_2
Callatostatin 2	Leu-Asn-Glu-Glu-Arg-Arg-Ala-Asn-Arg-Tyr- Gly-Phe-Gly-Leu-NH_2
Callatostatin 3	Ala-Asn-Arg-Tyr-Gly-Phe-Gly-Leu-NH_2
Callatostatin 4	Xaa-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH_2
Callatostatin 5	Gly-Pro-Pro-Tyr-Asp-Phe-Gly-Met-NH_2
Mas-AS	pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe- OH

Table 2.8: The sequences of the callatostatins and the structurally unrelated Mas-allatostatin.
Callatostatins 1 and 5 were tested in this study and found to be inhibitory in the larval heart.

Chapter 3

The Actions of Bioactive Peptides and Neurotransmitters on the Oviduct of *Manduca sexta*.

3.1 Introduction

The insect oviducts are the means by which eggs are transported from the ovarioles to the ovipositor prior to egg-laying. The muscular tubes move the eggs by spontaneous contraction but oviduct motility is likely to be additionally subject to the regulatory effects of innervation, circulating hormones and the exogenous secretions from the male insect introduced during mating. The nature of these controls over the spontaneous contractions remains largely unknown but these factors must act to orchestrate the activity of the whole of the reproductive system during reproduction including fine tuning the myogenic contractions of the oviducts.

Control of Contractile Activity in the Oviduct

The investigation of oviducal controls has been limited by the requirement for a means of measuring contractions in response to applications of putative regulatory substances. This necessitates the use of an *in vitro* technique. Oviduct bioassays have been developed in a limited range of insect species. In all insects tested to date, extracts from the abdominal nerve cord and brain have stimulated oviducal contractions. The responses of oviducts from Orthopteran species have been the most extensively studied however the results from these studies and the little evidence available from other species shows that factors controlling oviduct motility are not comparable between insect species. Data from other insects is largely limited to tests of biogenic amine neurotransmitter substances. The effects of neuropeptides other than proctolin have not been fully investigated. As examples of the differences observed between species, in the locust, octopamine has been implicated as a substance inhibitory to lateral oviduct contractions at physiological doses (Lange and Orchard 1986) but has been reported as both excitatory and inhibitory in the *Leucophaea maderae* (Cook *et al.* 1984) and excitatory in *Gryllus bimaculatus* at low doses (Sefiani 1987). L-glutamate has been shown to be excitatory in the locust, *Leucophaea maderae* and *Gryllus bimaculatus* but is not active in the oviduct of the stable fly *Stomoxys calcitrans* (Cook and Wagner 1991). Acetylcholine has been reported as stimulatory in *Leucophaea maderae* oviduct (Cook *et al.* 1984) but was inactive in *Gryllus bimaculatus* (Sefiani 1987). Proctolin has been reported as stimulatory in the oviducts of all species tested.

The *Locusta migratoria* and *Leucophaea maderae* oviduct bioassays have been employed as tools in the isolation of a number of myoactive neuropeptides (review: Schoofs *et al.* 1993). The peptide Lom-Ag-myotropin II isolated from locust male

accessory glands is active in the *Locusta* oviduct but not in that of *Leucophaea.* Other peptides active in the oviducts of *Locusta* include locustachykinins, locustapyrokinins, locustamyotropins, schistoFLRFamide, CCAP and Lom-AG-myotropin I. Clearly, the oviducts of *Locusta* are responsive to a large range of peptides. The physiological significance of multiple myotropic factors acting on the sane tissue is unknown.

The available data concerning oviduct motility is not extensive with a lack of information regarding insect oviduct regulation as a whole. The shortfall occurs both at the level of the range of species investigated but also in the number of neurotransmitters and in particular neuropeptides tested. Studies have centered almost exclusively on Orthopterans with only limited testing of the vast range of recently identified insect myoactive peptides.

The aim of this study is to extend the present knowledge of the regulation of oviduct contraction by developing a novel oviduct bioassay in the Lepidopteran *Manduca sexta.* The lack of an oviduct bioassay from this order has meant that the identity of neurotransmitters and neuropeptides involved in oviduct motility in these insects are unknown. Therefore I have sought to present as wide a range of neurotransmitters and neuropeptides as possible to the isolated *Manduca* oviduct and to compare the results to those of the better studied species, *Locusta migratoria.* The responses obtained from the *Manduca* oviduct indicate substances likely to play a role in oviduct motility and may identify myoactive peptides previously not detected in this species. A detailed study of the peptidergic and aminergic regulation of the oviduct contractions is an essential prerequisite to the understanding the neuroendocrine control of female reproduction in this moth.

3.2 Methods

3.2.1 The *Manduca* Oviduct Bioassay

The culture of the *Manduca sexta* used in this study has been described in Chapter II.

Female insects of the required stage were selected. The adults used were approximately 24 hours after eclosion and the pupae of 24 hours prior to eclosion. The abdomen was severed from the thorax and opened ventrally and pinned out onto a Sylgard dish. The abdominal nerve cord was removed and the preparation washed thoroughly. The cuticle around the ovipositor vent was cut freeing the common oviduct, the ovipositor and the associated tissues from the cuticle. This tissue mass was then lifted away and the muscle connecting the common oviduct cut away from underneath. The ovaries were removed leaving the lateral oviducts intact. As much fat body as possible was removed from the oviduct. Following dissection, the oviduct was pinned by the cuticular portion of the ovipositor into a narrow Sylgard surfaced channel in a perspex block. The whole block was orientated horizontally. Saline was run by gravity feed into a port at one end and exited via a similar port at the other end. A small (1ml) chamber before and after the channel ensured that a head of saline could be maintained covering the oviduct tissue. The cut ends of the lateral oviducts were tied with a length of cotton which was threaded under a loop attached to the channel base and attached to the force transducer arm (Palmer isotonic force transducer). Movements of the transducer arm were recorded with a chart recorder (Houston Omniscribe). The saline flow and the orientation of the chamber were adjusted such that the tissue was constantly covered by saline. The flow rate used was approximately

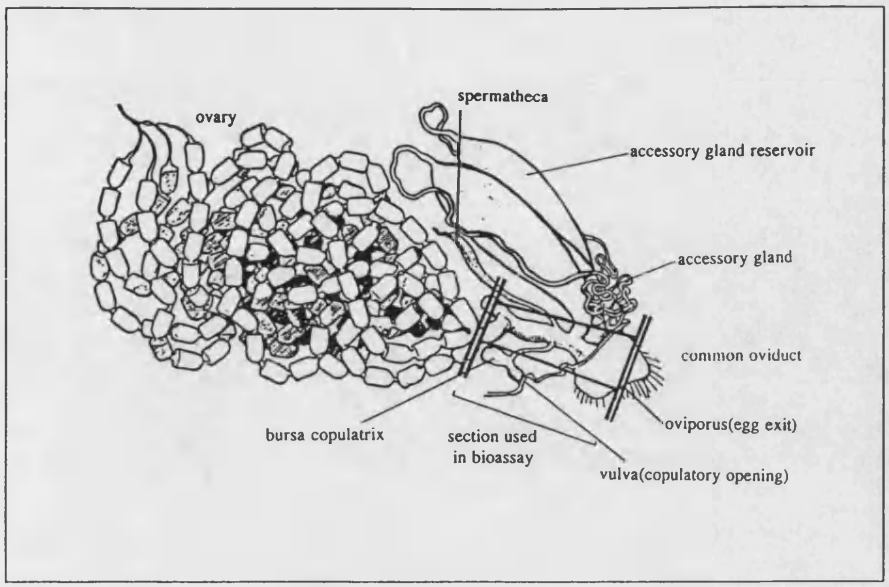

Figure 3.1: Diagram showing the portion of oviduct used in the *Manduca* oviduct bioassay.

40ml/hour. After preparation, it was necessary to allow the oviduct to wash for 2-4 hours. Spontaneous contractions usually commenced soon after dissection however a washing period was essential before the basal contraction rate became constant. Testing commenced once a steady spontaneous contraction rate of approximately 1 to 2 contractions in every 5 minutes was observed.

The portion of oviduct used in the oviduct bioassay is shown in Fig. 3.1 and the experimental setup is diagrammatically depicted in Fig. 3.2.

3.2.2 Experimental Procedure

Fresh test samples of peptide solutions were prepared daily from aliquots of 10^{-5} M stored in distilled water at -40°C. Neurotransmitter test samples were freshly prepared from a stock solution of 10^{-3} M in dH_2O and stored at +4°C. Serial dilutions were prepared in fresh adult *Manduca* saline.

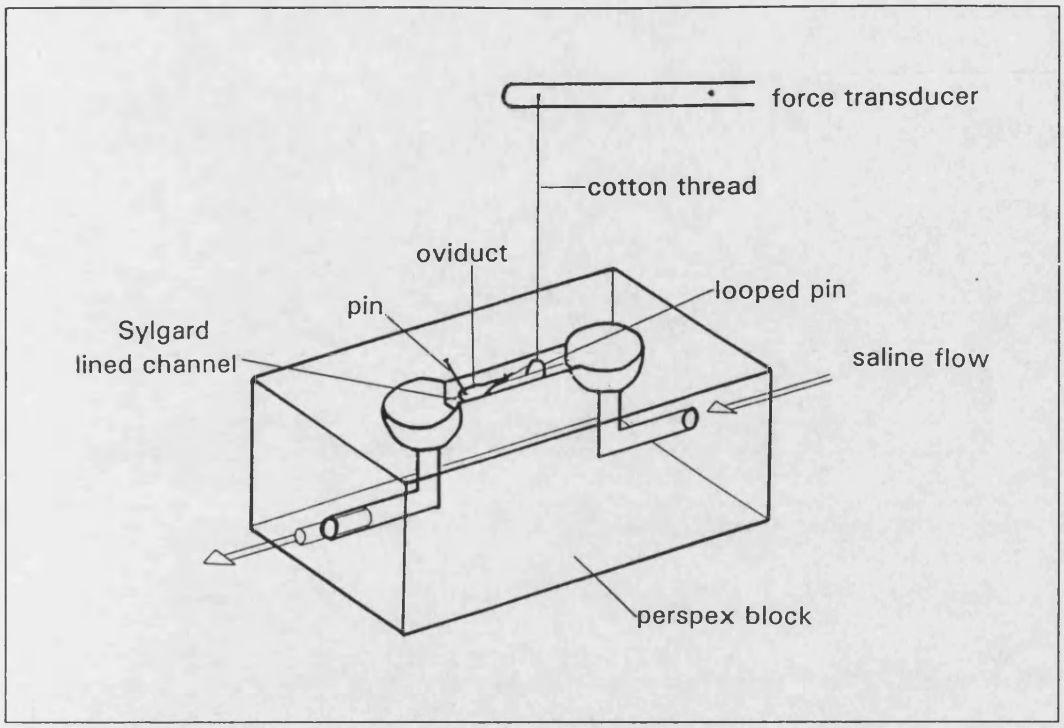

Figure 3.2: Diagram showing the setup of the oviduct bioassay.

Samples were applied in 100μl aliquots in adult *Manduca* saline directly into the saline flow 0.5cm in front of the tissue using a Gilson pipette. The concentration of the test substance at the oviduct was less than that stated because a pulse application of 100μl was made into a volume of approximately 1ml of saline contained in the channel of the perspex block. Further applications of samples were only made after the contraction rate had returned to the basal rate as before the previous application. Saline control applications were made throughout testing and standard doses of CCAP were used to determine the relative responsiveness of preparations. The chart speed used in all tests was 0.25cm/minute. All assays were performed at room temperature (22$\pm$2°C).

The response of the oviduct was quantified by comparison of the contraction rate during the five minutes following application with that of the five minutes preceding the application.

Peptides used were:-

- Crustacean cardioactive peptide (CCAP) (synthesised for this study)
- *Manduca sexta* allatotropin (Mas-AT) (Sigma)
- Locustatachykinin II (Lom-TK II) (synthesised for this study)
- FMRFamide (Sigma)
- FLRFamide (Sigma)
- SchistoFLRFamide (gift of Dr. P. D. Evans, University of Cambridge)
- ManducaFLRFamide (gift of Dr. T. Kingan, USDA, Beltsville, Maryland, USA.)
- Leucomyosuppressin (Lem-MS) (Peninsula)
- Corazonin (Sigma)
- *Heliothis zea* Pheromone biosynthesis acivating neuropeptide (Hez-PBAN) (Peninsula)
- Leucopyrokinin (Lem-PK) (Sigma)
- Proctolin (Sigma)

Neurotransmitters used were:-

- Acetylcholine chloride (ACh) (BDH)
- Cyclic adenylmonophosphate (cAMP) (Sigma)
- Adrenaline hydrogen tartrate (BDH)
- Dopamine (3-hydroxy-tyramine) (Sigma)
- Gamma amino butyric acid (GABA) (Sigma)
- DL-glutamic acid (Fisons)
- Noradrenaline (Sigma)

- Octopamine (Sigma)
- Serotonin (5-hydroxytryptamine, 5-HT) (Sigma)

3.3 Results

3.3.1 Neuropeptides

A range of known insect neuropeptides were applied to adult and pupal oviducts. The results are shown in Table 3.1. From those tested only the peptides CCAP and *Manduca sexta* allatotropin were found to be myoactive on the *Manduca* oviduct. The dose response curves of the oviduct to these peptides are shown in Fig. 3.3 and Fig. 3.6. Examples of the responses of the oviduct to CCAP and Mas-AT are shown in Fig. 3.4 and Fig. 3.7.

The peptide CCAP was the most potent myostimulator with a threshold of between 5×10^{-11} and 10^{-11} M in the adult oviduct. The maximum response gave a mean increase in contraction rate from one to 13 in five minutes. This represents an increase of 1200.0% over the basal contraction rate at a dose of 5×10^{-10} M. In the pupal oviduct the threshold concentration was ten fold more at 10^{-10} M. The differences between the adult and pupal oviduct responses are shown in Fig. 3.5. The results for comparison of the adult with pupal oviducts were obtained from a different preparation to those of the CCAP dose response curve.

The threshold concentration of Mas-AT was between 10^{-8} and 10^{-9} M with a mean increase in contraction rate from one in every five minutes to six in every five minutes at the maximum concentration tested of 10^{-6}M. The response of the pupal oviduct to Mas-AT was not tested.

The response profile of the oviduct to the two peptides was not identical, the differences can be seen when the responses shown in Fig. 3.4 and Fig. 3.7 are

compared. The application of CCAP at all concentrations greater than 10^{-11} M caused an initial rapid increase in the frequency of contractions, followed by a prolonged period of up to 20 minutes in which the frequency of contractions declined back to the original basal rate. In comparison allatotropin, at concentrations greater than the threshold, caused a rapid and short-lived response usually lasting less than a minute even up to high doses. Habituation of the oviduct to allatotropin was noted. Approximately 30 minutes of washing was required before a similar repeat response could be obtained. This effect was not noted for CCAP.

3.3.2 Neurotransmitters

The results from testing a range of neurotransmitters on the oviduct bioassay are shown in Table 3.2. Dose response curves were not constructed for any of the neurotransmitters because in all cases the threshold doses were too high to obtain useful results. The response of the oviduct and the minimum threshold dose required to elicit change in the basal contraction rate are given. The threshold doses were determined from at least three applications of the same concentration. Examples of the responses of the adult oviduct to some neurotransmitters are shown in Fig. 3.8.

The neurotransmitters dopamine, GABA and serotonin all stimulated an increase in the oviducal contraction rate at a concentration of 10^{-3} M. GABA did not effect the pupal oviduct at this concentration but only the adult.

The neurotransmitter glutamic acid had a threshold concentration of 10^{-4} M in both the pupal and adult oviducts.

At a dilution of up to 10^{-5} M, octopamine may have inhibited the spontaneous contraction rate in both the pupal and adult oviducts. Inhibition was not readily detectable in this bioassay due to the extended periods of inactivity of the oviduct between contractions and the relatively brief response time. In a number of preparations octopamine was observed to inhibit a spontaneous contraction when applied immediately before a normal contraction was expected. The response was not consistent but occurred in approximately 50% of tests. This effect was not seen in saline control applications.

None of the other neurotransmitters tested caused a response at concentrations of less 10^{-3} M.

Evaluation of the oviduct preparation

The *Manduca* oviduct bioassay proved to be simple and quick to prepare although some preparations required extended equilibration periods of two or more hours. The results obtained show that this bioassay is very sensitive to a number of myotropic factors and long-lived with consistent responses durng a day of testing.

The slow response and recovery times proved to be a major drawback with the bioassay. Responses to high doses required recovery times of up to one hour. Further applications of test substances could only be made after the basal contraction rate had been resumed from the previous test sample. With contractions only occurring once every four to five minutes this required a delay of at least eight minutes between applications even if no response was seen. This severely limited the number of samples which could be tested in a day.

3.3.3 Summary

A novel bioassay using the isolated oviduct of *Manduca* has been used to screen a range of neurotransmitters and neuropeptides. The conclusions from this survey are firstly, that a number of neurotransmitters stimulate contractions at high doses. Of these only glutamic acid and octopamine stimulate the oviduct at doses which are likely to be of physiological significance. Secondly only the peptides CCAP and Mas-AT are myoactive on the oviduct. The response to these two peptides was not the same and the pupal oviduct was an order of magnitude less responsive to CCAP than that of the adult.

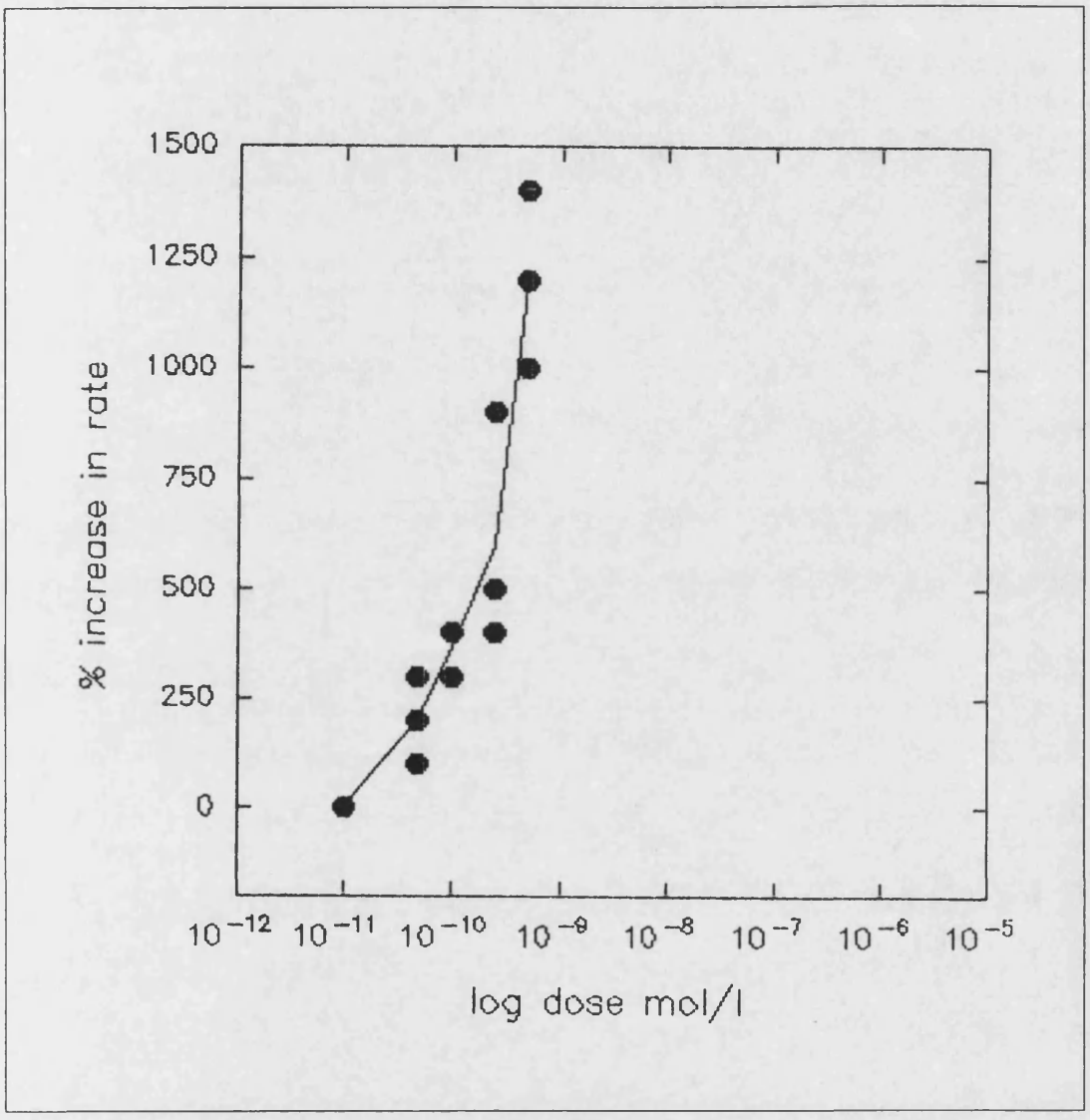

Figure 3.3: Graph showing the response of the adult oviduct to CCAP (crustacean cardioactive peptide).
The points represent the result of one application at the corresponding concentration. The curve shows the mean response to three applications. Applications were made in 100 µl of adult Manduca saline. The response rate was measured over the 5 minutes following the application and compared to the rate of the preceding 5 minutes.

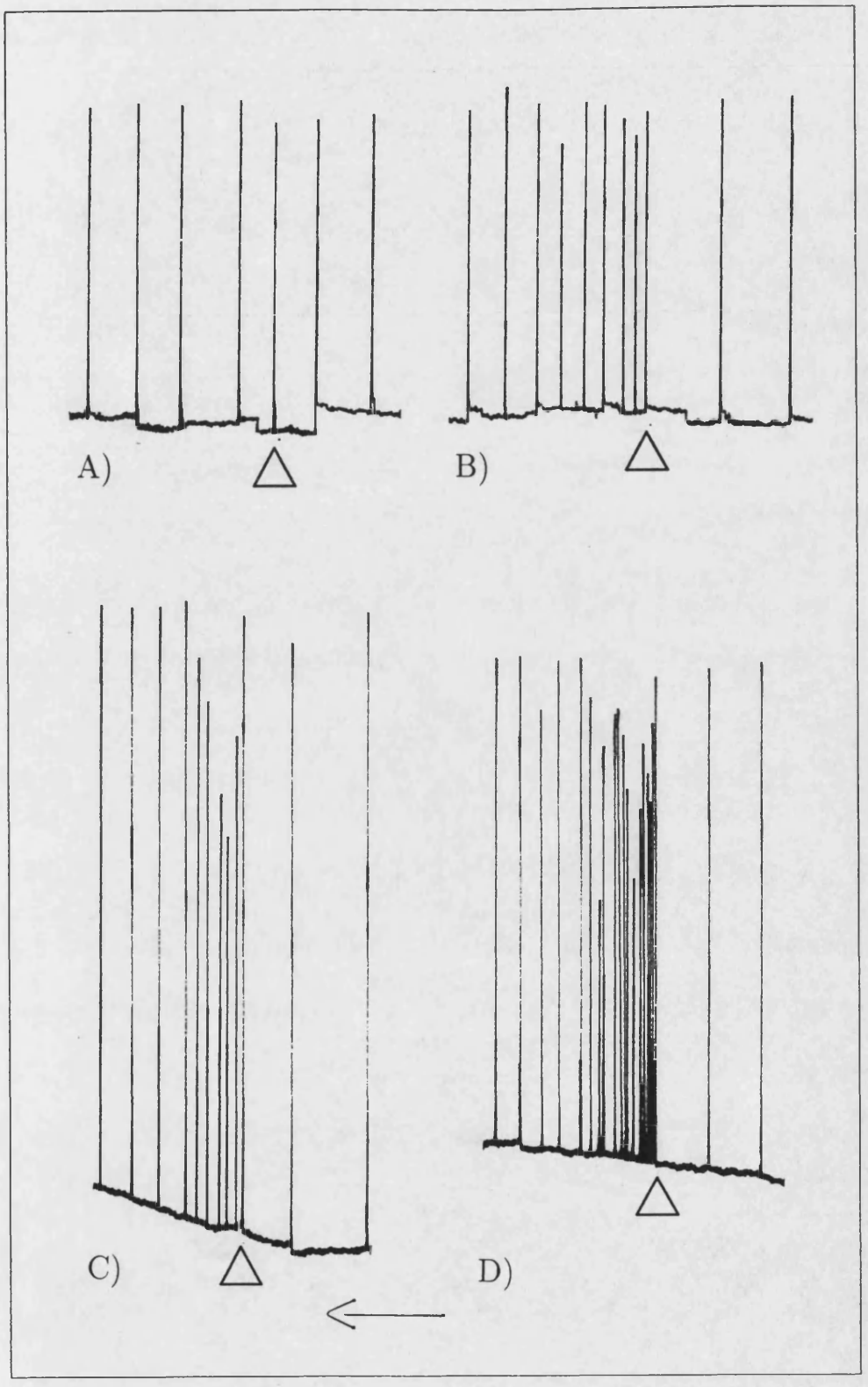

Figure 3.4: Representative examples of the response of the adult oviduct to CCAP. *A) 10^{-11} M, B) 5 x 10^{-10} M, C) 10^{-10} M, D) 5 x 10^{-9} M. Chart recorder speed was 0.25 cm/min.*

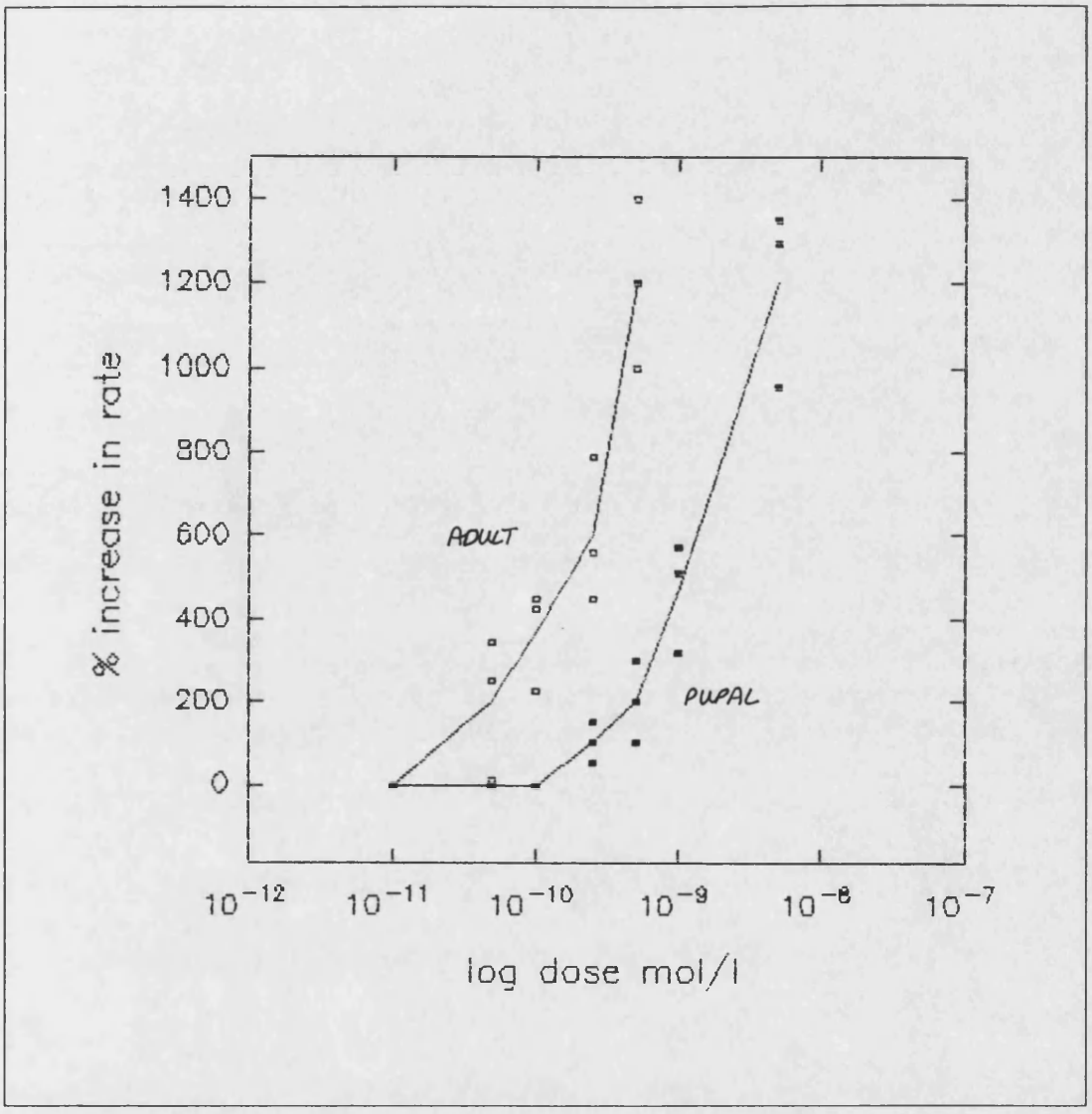

Figure 3.5: Graph showing the difference in response of the oviduct to CCAP of virgin adults 24 hours after eclosion and pupae 24 hours prior to eclosion.
Applications were made in 100 µl of adult Manduca saline. The response rate was measured over the 5 minutes following the application and compared to the rate of the preceding 5 minutes.

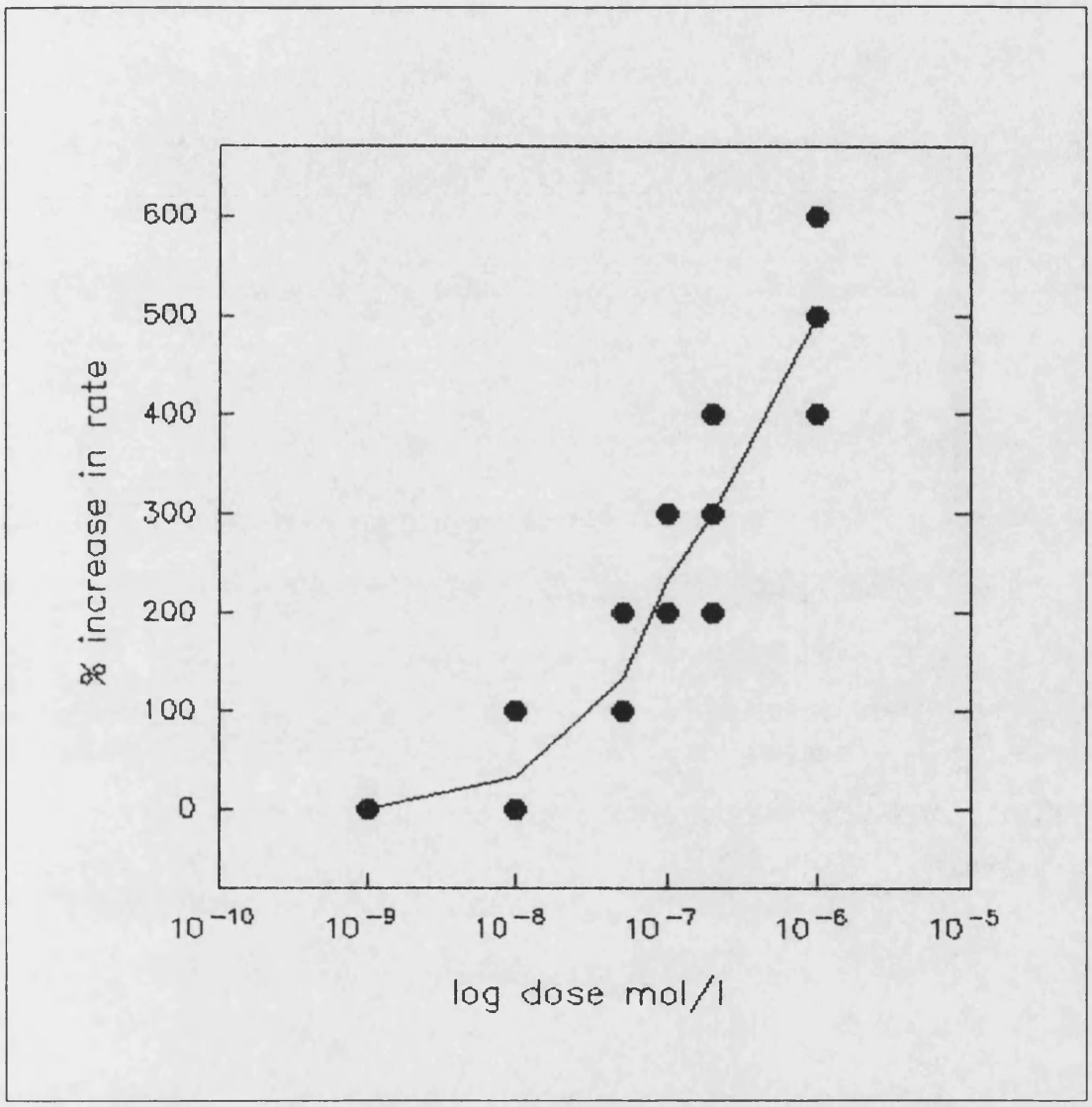

Figure 3.6: Graph showing response of the adult oviduct to *Manduca* allatotropin. *The points represent the result of one application at the corresponding concentration. The curve shows the mean response at that concentration. Applications were made in 100 µl of adult Manduca saline. The response was measured over the 5 minutes following the application and compared with that observed in the 5 minutes prior to the application.*

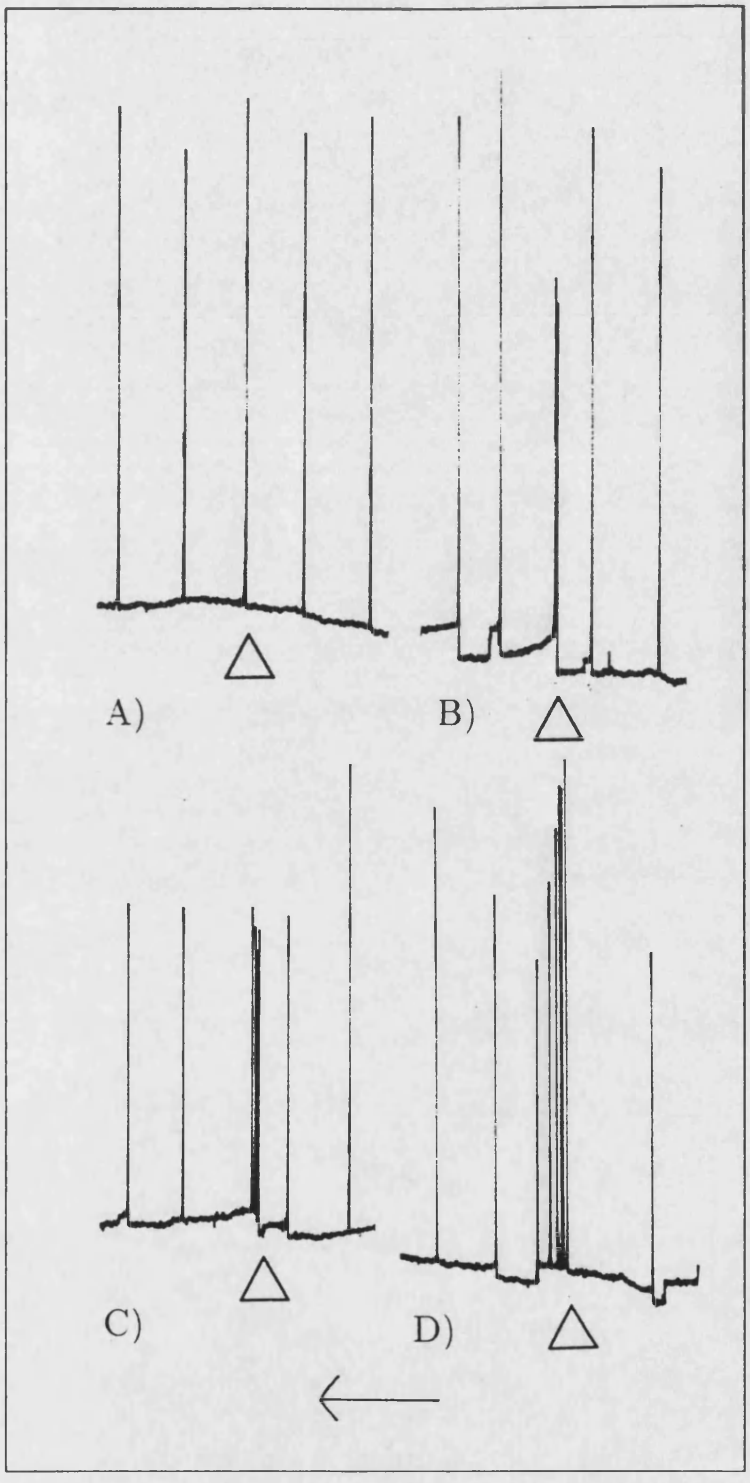

Figure 3.7: Representative examples of the responses of the oviduct to Masallatotropin.
A) 10^{-9} M, B) 10^{-8} M, C) 10^{-7} M, D) 10^{-10} M. Chart recorder speed was 0.25 cm/min.

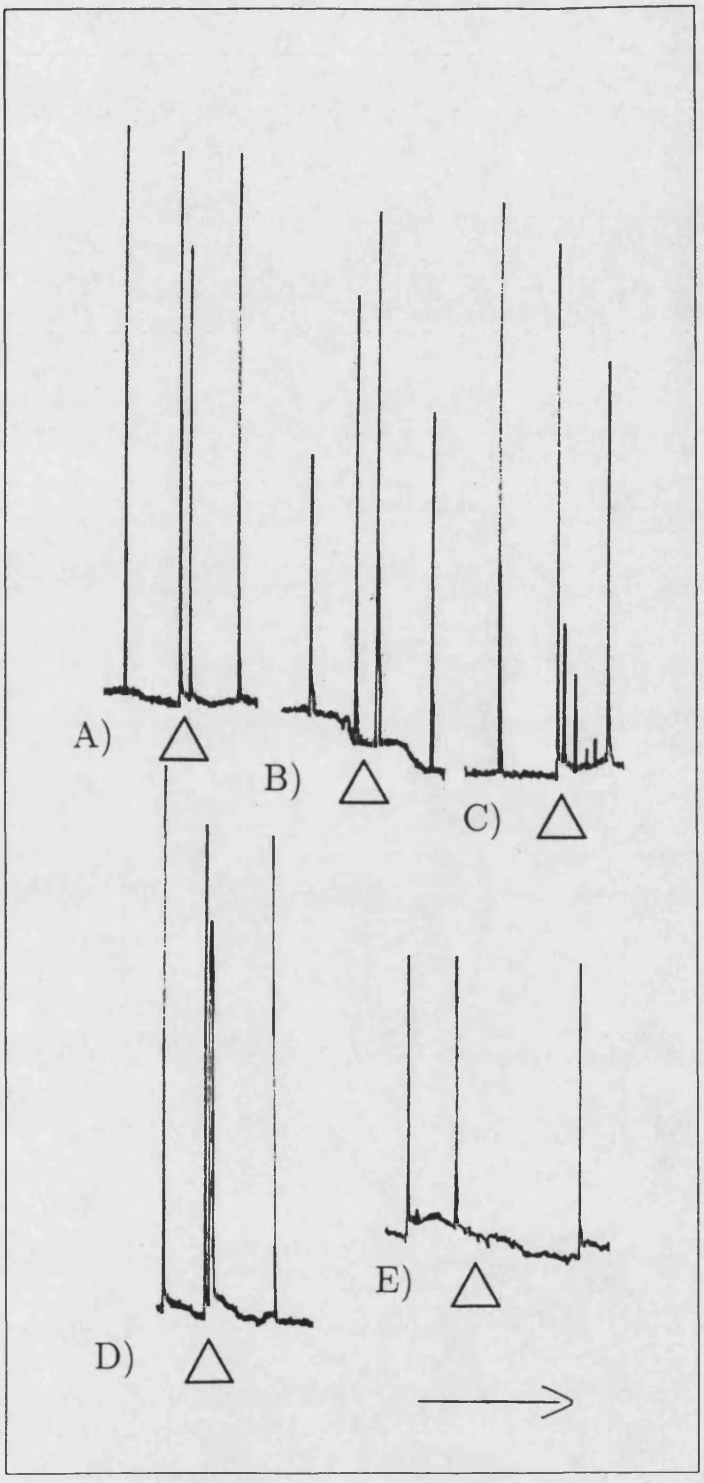

Figure 3.8: Representative examples of the responses of the adult oviduct to neurotransmitters.
A) dopamine 10^{-3} M, B) GABA 10^{-3} M, C) 5 HT 10^{-3} M, D) glutamic acid 10^{-4} M, E) octopamine 10^{-5} M. Chart recorder speed was 0.25 cm/min.

Peptide	Pupal Oviduct	Threshold Dose	Adult Oviduct	Threshold Dose
AKH	inactive		inactive	
Mas-allatotropin	active	10^{-8} M	active	10^{-8} M
CCAP	active	10^{-10} M	active	10^{-11} M
Corazonin	inactive		inactive	
FMRFamide	inactive		inactive	
Lem-PK	inactive		inactive	
Lom-TK II	inactive		inactive	
MasFLRFamide	inactive		inactive	
Hez-PBAN	inactive		inactive	
Proctolin	inactive		inactive	
SchistoFLRFamide	inactive		inactive	

Table 3.1: Table showing responses of the adult and pupal oviducts of *Manduca sexta* to a number of known myoactive neuropeptides

Neurotransmitter	Pupal Oviduct	Threshold Dose	Adult Oviduct	Threshold Dose
Acetylcholine	inactive		inactive	
Adrenaline	inactive		inactive	
cAMP	inactive		inactive	
Dopamine	active	10^{-3}M	active	10^{-3}M
GABA	active	10^{-3}M	inactive	
DL-glutamic acid	active	10^{-4}M	active	10^{-4}M
Noradrenaline	inactive		inactive	
Octopamine	active (inhibitory)	10^{-5}M	active (inhibitory)	10^{-5}M
Serotonin	active	10^{-3}M	active	10^{-3}M

Table 3.2: Table showing the response of the adult *Manduca* oviduct to a range of neurotransmitters

3.4 Discussion

The oviduct bioassay described in this study provides a novel technique for the detection of factors myotropic on this tissue. The development of this bioassay is of particular value because it is the only oviduct preparation reported in a Lepidopteran species to date. The bioassay forms a new screening tool for myoactive peptides in insects and will also aid in the understanding of the regulation of oviducal contractions in Lepidoptera *in vitro* and of the female reproductive system *in vivo*. The results obtained in this study are particularly interesting as they clearly show that the *Manduca* oviduct is not subject to the same neuropeptide controls as the considerably better known and more extensively studied oviduct of the Orthopteran, *Locusta migratoria*.

When a range of known neurotransmitters and neuropeptides were presented to the *Manduca* oviduct, octopamine, CCAP and *Manduca* allatotropin modulated the basal contraction rate. No other substance tested effected the oviduct at physiological concentrations. In comparison the locust oviduct has been shown to respond to a wider range of myotropic factors. Table 3.4 details the differences in the responses between the oviducts of the locust *Locusta migratoria* and *Manduca sexta*.

In the locust, proctolin, octopamine and extended FLRFamides in particular have all previously been implicated in the regulation of oviducal contractions *in vivo*. The locust oviducts are sensitive to low doses of proctolin with a threshold of between 10^{-11} M and 10^{-10} M (Orchard *et al.* 1989) and immunological evidence shows proctolin-like innervation of the oviducts in the locust (Eckert *et al.* 1989, Lange *et al.* 1986), strongly suggesting a regulatory role in the oviduct in this insect. Results from this study demonstrate that by contrast, in the

Manduca oviduct, proctolin is not myotropic at physiological levels. Although no investigation of proctolin immunoreactive fibres in the abdomen has been reported, immunological evidence has shown that Lepidpotera characteristically have, relative to other orders, few proctolin immunoreactive neurons. These are mainly located in the brain, frontal ganglion, suboesophageal ganglion and thoracic ganglia with few occurring in the abdominal ganglia (Davies *et al.* 1989). The distribution of proctolin and lack of response to this peptide in the oviduct bioassay indicate that proctolin does not play an important role in acting on the reproductive organs in Lepidoptera. This peptide is more likely to be involved in the stomatogastric nervous system. The lack of response to this peptide of both the heart (Chapter II this study) and the oviduct in *Manduca* supports this hypothesis.

Following the discovery of octopamine in the salivary glands of *Octopus* (Erspamer and Boretti 1951), this analogue of norepinephrine has since been found to be widespread in insect nervous tissue and have a broad range of targets and responses (Evans 1985). Octopamine is inhibitory in the locust oviduct, reducing both the rate and strength of the spontaneous contractions (Lange and Orchard 1986) at doses of 10^{-9} M. A subset of the dorsal unpaired median neurons in the 7th abdominal ganglion of the female locust are known to project to the oviduct and to be octopaminergic (Lange and Orchard 1984, Orchard and Lange 1985). In the oviduct of the stable fly *Stomoxys calcitrans* octopamine is inhibitory at doses of 10^{-8} to 10^{-6} M (Cook and Wagner 1992). This evidence points to octopamine being of importance in oviduct regulation in both the locust and the stable fly.

In *Manduca* results from this study suggest an inhibitory function of octopamine in the oviduct however the dose required for threshold was four orders of mag-

nitude higher than in the locust and a minimum of one order higher than the stable fly. Detection of inhibitory responses was not conclusive with this bioassay therefore the high concentration required to elicit a response may reflect an artefact of the bioassay method.

Octopamine is present in *Manduca* (Robertson and Juorio 1976, Klassen and Kammer 1985) and has also been found associated with the tissues of the oviduct (Davenport and Wright 1986). Octopamine is present in the terminal ganglia at elevated levels in comparison to the remaining abdominal ganglia. There have been no reports detailing octopamine innervation of the *Manduca* oviduct by immunohistochemical means which makes confirmation oviducal regulation by direct action of nerves on the tissues difficult. Measurements of octopamine levels in the haemolymph of adult *Manduca* were found to be 10^{-8} M in developing adults increasing to 10^{-7} M in ecdysed individuals by Klassen and Kammer (1985) but only 10^{-9} M to 10^{-8} M in adults by Prier *et al.* (1994). At either of these concentrations octopamine would not act as a blood bourne myoactive factor to regulate oviduct motility based on the results from this study. Some reports have suggested that octopamine may act to enhance nervous transmission rather than be active alone (Klassen and Kammer 1985, Prier *et al.* 1994). The response of the semi-isolated *Manduca* heart to the *Manduca* cardioactive peptides, the CAP2s is potentiated two-fold in the chronic presence of subthreshold concentrations of octopamine (Prier *et al.* 1994). CCAP, a member of the CAP2 peptides is myoactive in the *Manduca* oviduct. A similar mechanism involving the up-regulation of the response to neuropeptides in the presence of subthreshold levels of octopamine might occur in the oviducts however this possibility was not investigated in this study.

The neuropeptide schistoFLRFamide is a potent inhibitor of oviducal contrac-

tions in the locust with a threshold of 10^{-9} M to 10^{-8} M (Lange *et al* 1991) as is the structurally similar leucomyosuppressin (Lem-MS) from *Leucophaea maderae* (Peeff *et al.* 1993). Chromatographic and immunological evidence shows that schistoFLRFamide or structurally similar peptides are associated with the oviduct of the locust (Lange *et al.* 1991, Schoofs *et al.* 1993). Four other FLRFamide related peptides have recently been isolated from *Locusta* (Peeff *et al.* 1994, Lange *et al.* 1994). One of these peptides is closely related to schistoFLRFamide differing only in two positions and has a similar inhibitory effect on the oviduct. Two of the other three peptides have been tested on the locust oviduct and are stimulatory at a threshold of 5×10^{-10} M. The results suggest that a minimum of two types of receptor for FLRFamide-like peptides are present in the locust oviduct with one resulting in stimulation and the other causing inhibition. Characterisation of the inhibitory oviducal receptors for schistoFLRFamide has revealed the presence of two types of receptor for this one peptide, one of high affinity and the other of low affinity (Wang *et al.* 1994). These receptors show a regional distribution with the low affinity receptors present mainly in the innervated lower lateral oviduct and the high affinity in the upper lateral oviduct where innervation is lacking.

The low threshold doses and immunological evidence of FLRFamide-like innervation of the oviduct suggests that these peptides are important in regulation of locust oviduct motility. Variations in the N-terminus determine either a stimulatory or inhibitory action (Lange *et al.* 1994) and receptors to these peptides are present in different regions of the oviduct (Wang *et al.* 1994). A complex oviducal control mechanism by FLRFamide-like peptides in the oviduct of the locust is implicated involving several members of this peptide family.

In *Manduca* the structurally similar peptides schistoFLRFamide, Lem-MS and

manducaFLRFamide failed to elicit any response in the oviduct. Although the difficulty in detecting inhibitory responses may have masked such effects, no alteration in the basal contraction rate was noted following an application of these peptides. This evidence suggests that FLRFamide-related peptides do not play a regulatory role in the oviduct of *Manduca sexta.* This finding is further supported by the absence of immunoreactive fibres terminating on the oviduct (A. K. Marshall and K. S. J. Thompson, unpublished) detected with a schistoFLRFamide specific antiserum. Clearly FLRFamide related peptides are not as important in *Manduca* oviduct motility as they are in in the locust oviduct.

A number of other myoactive factors are known to influence contractions of the oviduct of the locust however the physiological role of these *in vivo* is less well supported by experimental evidence.

The neuropeptide CCAP has potent myotropic effects on the oviduct of the locust (Stangier *et al.* 1989). The oviduct in this insect is not innervated directly by CCAP immunoreactive fibres (Dircksen *et al.* 1991) however CCAP released into the circulating haemolymph via the perisympathetic organs may affect oviduct motility. CCAP is strongly myotropic in the *Manduca* oviduct with a similar threshold to that in the locust (Dircksen *et al.* 1991). Unlike the evidence from the locust, in *Manduca* CCAP immunoreactivity is seen in the nerves extending to the oviduct (this study Chapter IV) with anti-CCAP immunostaining showing neurohaemal areas along specific caudal nerves of the terminal ganglia. CCAP is likely to act as a circulating neurohormone in the regulation of the *Manduca* oviduct. The generalised pattern of staining in the nervous system is not the same in the two insects. CCAP immunoreactive neurons in the locust are more numerous in ventral lateral regions of the terminal ganglia (Dircksen *et al.* 1991) whilst in *Manduca*, CCAP immunoreactive neurons largely originate in the me-

dian parts of the terminal ganglia (Davies *et al.* 1993). The relevance of these neuron distribution differences may signify a dissimilarity in the role of CCAP in these two insects.

The pupal oviduct was an order of magnitude less responsive to CCAP than the unmated adult oviduct. Two explanations are possible; the muscle of the oviduct may not be fully developed in the pupa and therefore not as contractile or the pupal oviduct may express less CCAP receptors than the adult. The adult female moth mates on the first night after eclosion and eggs are laid on the following nights (Sasaki and Riddiford 1984). The number of receptors for CCAP may increase during this time reaching a peak at the time of egg-laying. There was no difference detected in the sensitivity of the mated and unmated female oviducts to CCAP in this study.

All the members of the family of Lom-TKs so far isolated are myotropic in the locust oviduct at physiological concentrations (Schoofs *et al.* 1990a,c) as are the pyrokinins and pheromonotropins (Schoofs *et al.* 1993d). This demonstrates that the locust oviduct has receptors to both the tachykinin and the kinin C-terminals and members of these two families are possibly important in oviduct motility. In comparison the *Manduca* oviduct did not respond to Lom-TK II, Lem-PK or Hez-PBAN. Although the peptide range tested was limited and therefore results could not be considered as conclusive this suggests that structurally similar peptides are not involved in oviduct regulation in this insect. The lack of response of the *Manduca* oviduct to the tachykinin C-terminal is somewhat surprising because this family of peptides appears to have been well conserved both in structure and physiological properties both in vertebrates and invertebrates. All vertebrate tachykinins identified stimulate smooth muscle in vertebrates and insect tachykinins stimulate the analogous visceral muscle in insects. The larval heart

of *Manduca* is sensitive to Lom-TK II but the adult heart is not (Chapter II). If tachykinins are present in *Manduca* this would indicate a change of role during metamorphosis as immunological evidence for the presence of a Lom-TK II-like peptide has been observed in the abdominal nerve cord of both the larva and the adult moth (A. K. Marshall, unpublished observations). That the *Manduca* oviduct does not have receptors to these peptides is unexpected in view of the known conserved effects of this peptide family.

The neuropeptide Mas-AT is myotropic in the oviduct of *Manduca* and this myoactivity suggests that this or a peptide of similar sequence is important in control of oviducal motility in this insect. Mas-AT stimulates juvenile hormone (JH) biosynthesis *in vitro* in the corpora allata of adult moths (Kataoka *et al.* 1989). Egg maturation is dependent on JH titre (Nijout and Riddiford 1979). Sasaki and Riddiford (1984) suggested that insertion of the spermatophore into the bursa copulatrix of the female at mating sends a nervous signal to the brain resulting in the maintenance of allatotropin secretion and hence JH biosynthesis and egg maturation. The results from this study suggest that Mas-AT may, in addition, have a direct action in the stimulation of oviduct musculature and that this peptide itself may be involved in female reproduction in *Manduca.*

Interestingly, Mas-AT bears a close sequence resemblence to one of the myotropins isolated from the accessory glands of *Locusta migratoria*, Lom-AG- myotropin I (Lom-AG-MT I) (Paeman *et al.* 1991) as shown in Fig. 3.3. Ten amino acid residues from a total of 13 are identical. The locust oviduct is 1000 times more responsive to Lom-AG-MT I than the hindgut suggesting specificity for the oviduct. The similarity of Mas-AT to Lom-AG-MT I could indicate the presence of a closely related peptide in the accessory glands of the male *Manduca* moth. Evidence of peptide transfer from male to female during mating and then, subsequent

alterations in female behaviour, has been noted in Dipterans (Chen *et al.* 1988, Chen and Balmer 1989) and some evidence of male derived oviduct myoactive peptides in the spermatophores of mated *Locusta* females has been seen (Schoofs *et al.* 1993d). The presence of male peptides in the reproductive tract of mated Lepidopteran females has not been shown. Stringer *et al.* (1985) concluded that the switch from female virgin calling behaviour to mated ovipositional behaviour in *Manduca* was mediated by sperm and/or seminal fluids from the male. T. M. Stephens (personal communication) found some evidence of oviducal stimulating peptides other than CCAP, in extracts of the accessory glands of *Manduca* however, as these were cardioactive in the larval heart they are not likely to have been Mas- allatotropin. Past work and the present study point to the importance of Mas-AT or a structurally similar peptide in female reproduction in *Manduca* but the presence, location and exact role are as yet uncertain.

Veenstra *et al.* (1993) suggested that Mas-AT is one of the CAP1s, a set of two cardioactive peptides partially isolated from adult *Manduca* but not as yet sequenced. A monoclonal antibody supposedly directed against common epitopes of the CAP1s and CAP2s (Taghert *et al.* 1983, 1984) stained a subset of the median neurosecretory cells in abdominal nerve cord ganglia (Tublitz and Sylwester 1990). An anti-allatotropin antiserum also stained a number of the same neurones and was reported to abolish all cardioactive effects in an HPLC fraction putatively containing CAP1 (Veenstra *et al.* 1994). In addition, Mas-AT, whilst a potent cardiostimulator in the adult has no effect in the larval heart. This evidence taken together points to Mas-AT being a CAP1. The CAP1s are released together with the CAP2s during emergence from the pupal case (Tublitz and Truman 1985c, Tublitz and Evans 1986) and during periods of flight in the adult (Tublitz 1989). Mating and in particular egg-laying behaviour are associated with flight episodes in the female moth giving a possible link between Mas-AT

Mas-allatotropin	Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe-	NH_2
Lom-AG-myotropin I	Gly-Phe-Lys-Asn-Val-Ala-Leu-Ser-Thr-Ala-Arg-Gly-Phe-	NH_2

Table 3.3: The sequences similarities of *Manduca* allatotropin and *Locusta* accessory gland myotropin.

release and reproductive behaviour in this insect.

Evidence from the HPLC separation of *Manduca* nerve cord in Chapter V of this study suggests that Mas-AT does elute at a similar time to the CAP1 peptides but if Mas-AT is indeed a CAP1 it may be only a minor component of the total CAP1 activity. A screening of the fractions obtained from an HPLC separation of adult nerve cord extracts shows that the CAP1 fraction is not stimulatory on the oviduct (Chapter IV), indicating that peptides other than Mas-AT must account for the cardiostimulatory activity of this fraction.

3.4.1 Summary

A *Manduca* oviduct bioassay has been developed and used to screen a range of factors known to be myoactive in insects. The bioassay forms a useful and viable technique for this purpose. From a range of known myotropic substances the *Manduca* oviduct responded to CCAP, *Manduca sexta* allatotropin and octopamine. The responses of the *Manduca* oviduct to various peptides were not the same as those reported for the locust oviduct. The *Manduca* oviduct provides a novel tool to aid in the identification of endogenous cardioactive peptides yet to be isolated from this insect.

Neurotransmitter/ Neuropeptide	*Locusta migratoria* Oviduct	*Manduca sexta* Oviduct
Acetylcholine	active	inactive
DL-glutamic acid	active	active
Octopamine	active (inhibitory)	active (inhibitory)
Serotonin	active	active
CCAP	active	active
SchistoFLRFamide	active (inhibitory)	inactive
Lem-PK	active	inactive
Lom-TK II	active	inactive
Hez-PBAN	active	inactive
Proctolin	active	inactive

Table 3.4: Table showing a comparison of the responses of the oviduct of *Locusta migratoria* with those of the *Manduca* oviduct to a range of neurotransmitters and neuropeptides

Chapter 4

Physiological Regulation of the Contractile Activity of the Oviduct in *Manduca sexta*.

4.1 Introduction

The regulatory systems governing the motility of the Lepidopteran oviduct and their relationship to reproductive behaviour are not well understood. The oviducts undergo continuous myogenic contractions as described in previous chapters, but additional mechanisms must be responsible for overall modulation and co-ordination of the reproductive system leading to oviposition. A knowledge of the factors governing oviducal contractions may aid in the understanding of Lepidopteran reproduction as a whole.

The three possible sources of regulation of oviducal motility are neural, hormonal and male derived factors. Investigation into the neural anatomy of adult *Manduca* reveals that the reproductive system is extensively innervated via nerves from the fused terminal ganglia (Thorn and Truman 1989) indicating neural involvement in oviducal regulation. As the oviducts are continually bathed in haemolymph, circulating neurohaemally derived hormones are also very likely to play a role in

regulation. The male moth transfers factors derived from the testes and possibly the accessory glands to the female during copulation which act on the female causing a switch from virgin calling behaviour to oviposition (Stringer *et al.* 1985). Male derived factors may act directly on the oviducts to modulate contractions or indirectly by stimulation of the release of neurosecretions.

The relative importance of these control mechanisms during the various stages of reproduction is not known. A study of the nerves and their influence in reproduction would be a valuable step forward in the elucidation of the mechanisms of Lepidopteran reproduction.

In Chapter III of this study the neuropeptides CCAP and allatotropin were implicated in the regulation of the oviduct because of their ability to influence the *in vitro* oviducal contractions. The purpose of this study was to further the previous investigations towards the identification of the factors governing *in vivo* oviducal motility in the female moth with the specific aim of confirming the involvement of these peptides. A detailed study of the pattern of innervation of the female reproductive system was carried out furthering studies made by other workers and allowing the identification of the terminal nerves responsible for the modulation of the contractions observed in the bioassay. Individual nerve pairs were dissected free, extracts were prepared and tested for myogenic activity in the larval heart and oviduct bioassays thus determining the presence of myoactive factors in specific nerves. The HPLC separation of these extracts and immunohistochemical staining for specific neuropeptides allowed confirmation of both the location and identity of the peptides.

4.2 Methods

The insects used in this study have been described previously in Chapter II.

4.2.1 Abdominal Nerve Cord

Extracts of Adult and Larval Nerve Cords

Insects of the required age were selected. In the adult, after removing the head and thorax, the abdomen was opened dorsally and the gut, reproductive organs and fat body were dissected free. The abdominal nerve cord could be lifted from the body wall intact supported by the dorsal pad. For larvae, 5th instar just prior to wandering were selected and anaethsetised by immersion in dH_20 at room temperature for approximately 15 minutes. The insect was opened dorsally and the gut removed. The nerve cord could then be lifted away from the body wall intact.

For crude extract applications to bioassays, nerve cords were pooled and homogenised using a hand-held glass-glass homogeniser in chilled *Manduca* adult or larval saline. The homogenate was centrifuged for 3 minutes in a microfuge (Eppendorf 5415 microfuge) and then the supernatant was boiled using a water bath for three minutes.

HPLC Separation of Abdominal Nerve Cord Extracts.

For HPLC separation the nerve cords were pooled and frozen at -40°C with a few crystals of PTU (phenylthiurea) to inhibit tyrosinase activity. When required the cords were homogenised in a chilled extraction medium of; 1M glacial acetic acid (BDH, HPLC grade), 20mM sulphuric acid (BDH, Analar grade), 0.1mM PMSF (phenyl methyl sulphonyl fluoride) in methanol (BDH, HPLC grade) and 1mM EDTA (ethylenediaminetetracetate disodium salt, Sigma) diluted to concentration in HPLC grade dH_2O. Ten nerve cords were homogenised in every 1ml of extraction media. The extract centrifuged for five minutes in a microfuge and the supernatant retained, the precipitate was resuspended in HPLC grade dH_2O, recentrifuged and pooled with the previous supernatant.

A SepPak (Waters, C-18) was previously primed with; 5ml HPLC dH_2O, 5ml acetonitrile (CH_3CN, Rathburns HPLC grade S), 5ml HPLC dH_2O then 5ml 10% acetonitrile/0.1% TFA (trifluoroacetic acid, BDH, HyPerSolv HPLC grade) using a luer fitting syringe. The sample was loaded onto the SepPak which was then washed with 2ml of the 10% acetonitrile/0.1% TFA. The SepPak was eluted with 2ml of 80% acetonitrile/0.1% TFA and the eluate lyophilised overnight.

The HPLC system is described in detail in Chapter V. The Reodyne injector was equipped with a 5ml injection loop. The column used for the HPLC separation was a C-4 Hypersil 10μm (25cm) with a C-4 Hypersil 10μm guard column (5cm). The solvents used were; HPLC grade dH_2O (Millipore filtered and double distilled) with 0.1% TFA and 100% acetonitrile also with 0.1% TFA. Mobile phase was pumped at 1ml/minute. The gradient used was: 0-5 minutes 10%, 5-65 minutes 10-70%, 65-70 minutes 70% and 70-75 minutes 70-10%.

The lyophilised nerve cord extracts were redissolved in approximately 1ml of 10% acetonitrile/0.1% TFA. This and washings adding up to about 4ml were injected into the system with the column equilibrated in 10% acetonitrile/0.1% TFA. The gradient was started only after the absorbance measured at 210nm dropped back to the basal level (ie. that seen previous to the injection). Fractions of 1ml each were collected from 5-65 minutes during the run.

Immediately after the completion of the HPLC run, aliquots of 200μl were taken from each of the fractions and lyophilised with 20μl of a 0.1% BSA (bovine serum albumin, Sigma heat shock fractionated) solution in dH_2O. This acted as a carrier to the semi-purified peptides thus minimising adsorbtion losses. Blanks containing 40% acetonitrile/0.1% TFA and 0.01% BSA were also prepared and lyophilised in parallel. The dried fractions were stored at -40°C until required and dissolved in *Manduca* saline prior to use in the bioassays.

Samples were applied to the bioassays at 0.25 insect equivalents for the larval and adult heart bioassays and 0.5 insect equivalents in the oviduct bioassay.

Extracts of the Terminal Ganglia Nerves.

Insects of the required age and sex were selected. The terminal ganglia and individual nerves were dissected out with the ganglia and matching pairs of nerves retained separately. The matching nerve pairs and ganglia were pooled in chilled distilled water. Lysis of the nerves was achieved by a repeated freeze-thaw action that is, freezing at - 40°C and thawing at room temperature a minimum of three times.

In initial experiments *Manduca* saline of ten times usual concentration was used to bring the samples to the normal saline strength prior to application to both the larval heart and adult oviduct bioassays. A dose volume giving the equivalent of 0.25 insects was applied to the larval heart bioassay and of one insect equivalent to the oviduct bioassay.

In further experiments, the terminal nerves and ganglia were collected as previously from ten pharate adult female *Manduca*. Following lysis in distilled water and repeated freeze thaw action, the solution containing each set of tissues was brought to 10% acetonitrile/0.1% TFA. The samples were then separated by HPLC using a C-4 column (Hypersil 10μm) as in previous experiments and a 10% to 40% acetonitrile/0.1% TFA gradient over 30 minutes with a 1 ml minute^{-1} flow rate. Fractions were collected every minute from 10% to 35% acetonitrile/0.1% TFA. The fractions were lyophilised overnight with 20μl of 0.01% BSA in dH_2O added to each.The fractions were redissolved in 500μl of adult saline and tested on the larval heart bioassay at an approximate dose of 0.8 insect equivalents and the adult oviduct bioassay at doses up to 1 insect equivalent.

4.2.2 Visualisation of the Oviducal Nerves

Methylene Blue Stain

Methylene blue was used as a nerve specific stain in order to visualise and aid in the tracking of the nerves from the terminal ganglia to the female reproductive tract.

An intra-vitam stain was used of reduced methylene blue. A 10.5% aqueous

methylene blue solution was acidified with a few drops of concentrated HCl, then filtered and heated to 80°C. Sodium formaldehyde sulphoxylate (Rongalite C, BDH) was added dropwise until the solution changed from blue to straw yellow. After cooling,the solution was filtered and allowed to stand for 24 hours before use directly on tissue under saline. Five preparations were treated by this method.

Methylene blue solution at 0.1% in *Manduca* saline was also used for staining. This was either applied directly onto tissue for up to five minutes under saline or injected into insects just prior to dissection in a volume of 200-300μl. A further five preparations were treated by this method. The results of the ten methylene blue stained preparations were compared and found to be very similar.

Cobalt Nerve Fills

Cobalt nerve fills were performed using a method adapted from Bacon and Altman (1977).

Insects were partially dissected under *Manduca* saline. The nerve to be filled was severed and a small vaseline cup formed around the cut end. Distilled water was added to the cup and left for a few minutes in order to lyse the severed nerve ending. The vaseline cup was then drained and refilled with a 1-3% cobalt chloride (CoCl) solution. The open top of the cup was then sealed with a cap of vaseline and the whole preparation submerged under saline was incubated in a humid chamber for up to 72 hours at 4°C.

After incubation the vaseline cup was carefully removed intact and the filled tissue fully dissected free of fat body, and removed from the insect carcass. The tissues

were then immersed in a 0.1% ammonium sulphide solution in insect saline for ten minutes followed by three to four rinses in saline. The preparation was fixed in Bouins fixative for one hour then washed in 70% alcohol and rehydrated down an alcohol series into distilled water. Timms developer was prepared previously; 3g gum acacia, 10g sucrose and 0.8g citric acid were dissolved in 100ml of dH_2O at 60°C then filtered through muslin. Immediatly prior to use 0.17g of hydroquinone was added and the solution brought to pH 2.6 with concentrated nitric acid. Each specimen was put into a solid base watchglass and 2ml of the developer added to each in an oven at 60°C. After one hour a fresh aliquot of the developer solution was added plus 200μl of a 0.1% silver nitrate solution. The specimens were handled with wooden cocktail sticks during this procedure. Incubation with the silver nitrate was carried out at 60°C, taking 20 to 60 minutes. The reaction could be followed by low powered microscope and the tissue transferred to dH_2O water when sufficient colour had developed. Following silver staining the tissues were pinned out on a Sylgard dish and dehydrated through an alcohol series. After clearing in methyl salicylate, the tissues were mounted in Canada balsam. The fills made were; 15 backfills of the nerve DN8, ten forward fills of DN8, five backfills of VN7 and five forward fills of VN7.

Immunohistochemistry

The anti-CCAP primary antiserum (2TB) used in this study was a kind gift of H. Dircksen (Dircksen and Keller 1987).

Four whole mount preparations consisting of the abdominal nerve cord, the terminal nerves and the female reproductive system were stained with the anti-CCAP antiserum. Insects were dissected being left fairly intact but tissues not required

were removed. The partially dissected insect was immersed in 4% paraformaldehyde or Bouins fixative at room temperature for 1 hour. The tissues were washed a minimum of three times in PBS (0.1M phosphate buffered saline pH 7.3), then usually left overnight in PBS at 4°C on a shaker. The tissues were then taken through an ethanol series (30, 50, 70, 85, 95 and three × 100% ethanol) with approximately ten minutes in each, followed by 30 minutes in methyl salicylate. The preparations were brought back down through the alcohol series into PBS with five minutes in each alcohol. The required tissues were dissected out removing as much of the fat body as possible and were blocked with 0.2% Triton/0.2% BSA/2–10% goat serum in PBS at room temperature for 30 to 60 minutes then washed in PBS. The primary anti-serum anti-CCAP 2TB was used at a concentration of 1 in 2500 to 1 in 3000 in PBS + 0.2% Triton (PBT) and incubated for three hours or overnight at 4°C on a shaker. The tissues were washed four times for 30 minutes in PBS then the secondary antibody (Vectorstain biotinylated goat anti- rabbit) was added at the required concentration. The tissues were soaked in 0.1% hydrogen peroxide in methanol for ten minutes, washed thoroughly in PBS and left to stand for 30 mins. PBS plus 0.1% Tween 20 rather than Triton was used for the following steps. Vectorstain ABC reagent was prepared as instructions and left to stand for 30 minutes (two drops of A into 10ml of buffer then two drops of B, mix well). ABC reagent was added to the samples and incubated at 4°C overnight. The tissues were washed in PBS, and using a section mould tray each sample was put separately into 1ml of PBT. 200μl of a 1mg/ml diaminobenzidine (DAB) solution in PBS was added and left to stand in the dark for one hour, allowing the DAB to permeate the tissues. 100μl of 0.4% H_2O_2 in water was added to each sample for ten minutes or longer. The progress of the reaction could be followed by eye and quenched when appropriate by washing in water.

The wholemounts were dehydrated through an ethanol series into xylene and mounted in Canada balsam (BDH).

4.3 Results

4.3.1 Abdominal Nerve Cord

Extracts of the Abdominal Nerves Cords of Adults and Larvae.

The abdominal nerve cord has been shown to contain factors which influence the motility of the oviduct. The location and nature of the endogenous neural controls governing oviducal contractions were investigated employing the oviduct bioassay as described in Chapter III.

The adult *Manduca* oviduct was found to have a threshold reponse to a crude adult abdominal nerve cord extract of approximately 0.025 nerve cord equivalents *in vitro*. The threshold response value did not vary according to either the age or the sex of the nerve cord donor insect. Typical recordings showing the similarity of the response to larval, adult female and adult male nerve cord extracts are shown in Fig. 4.4. Fig. 4.5 shows the response to various concentrations of nerve cord extract from pharate adults. The response of the oviduct to less than 0.5 insect equivalents of adult nerve cord extract was usually similar to the pattern seen in 2), 3) and 4) of Fig. 4.5. The initial rapid response would be shortlived and followed by an inactive period of approximately one minute then a further set of contractions would occur often less powerful than those of the primary response. The basal contraction rate would resume after four to six minutes.

Applications of greater than 0.5 insect equivalents did not show this pattern but rather a continuous elevation of contraction rate and the basal contraction rate resuming after approximately 10 to 15 minutes.

HPLC Separation of Abdominal Nerve Cords Extracts

Extracts of abdominal nerve cords of both pharate adults and larvae at one day prior to wandering were separated by HPLC. The resulting fractions were applied to the semi-isolated larval heart, the semi-isolated adult heart and the isolated oviduct bioassays. The percentage acetonitrile at which the active fractions eluted is an approximate figure allowing for the delay in the system between the pumps, the column, the detector and the fraction collecter.

The larval heart responded to fractions 25 – 27 from the adult nerve cord extracts (approximately equivalent to a acetonitrile/TFA concentration of 25 – 27%). The larval heart was responsive to fractions 25 – 26 from the larval nerve cord extracts (25 – 26% acetonitrile).

In the adult heart bioassay the fractions 26 and 28 – 29 from the adult nerve cord extracts were cardioactive (26% and 28 – 29% acetonitrile) and 25 – 26 from the larval nerve cord extracts (25 – 26% acetonitrile).

Myoactivity was detected by the oviduct in fractions 25 – 26 (25 – 26% acetonitrile) from the adult nerve cords and 26 from the larval nerve cord extracts (26% acetonitrile).

The responses of the three bioassays to the HPLC fractions of the adult nerve

cords are shown in Fig. 4.6, Fig. 4.7 and Fig. 4.8.

Terminal Nerves

In initial experiments, matching pairs of terminal nerves from ten unsexed pharate adults were pooled, lysed and tested directly for myoactivity on the larval heart bioassay and the adult oviduct bioassay. Figure 4.9 shows the response of the larval heart to 0.25 and 0.50 insect equivalents of each of the terminal nerve extracts together with ganglion 5 and the fused terminal ganglia extracts. Figure 4.10 shows the results in graph form. The relative myoactivity per weight of tissue was not assessed because the small size of the tissue caused difficulties during handling with the risk of tissue loss during weighing. The terminal nerves were all found to be of a roughly similar size with a descending size order of TN8 > VN8 > DN8 > VN7 > PN. The myoactivity in the nerve extracts did not correlate with the size ordering and the terminal ganglia sample contained considerably more tissue than terminal nerve samples but was found to be less myoactive. The lack of a measure of tissue mass meant that a direct comparison of specific myoactivity was not possible between samples, however the results clearly show that the extract of the terminal nerve DN8 was most cardioactive on the larval heart bioassay, 0.25 insect equivalents evoking a 165.0% increase over the basal heartrate. The terminal ganglia, VN7 and VN8 extracts all gave similar increases in heartrate of approximately a third that of the DN8 sample at the same dose. The TN8 extract gave an increase of 30%. The PN extract was not cardioactive.

The oviduct bioassay was only responsive to the DN8 and terminal ganglia extracts. The DN8 sample caused a 200% rate increase, twice that of the terminal

ganglia.

The distribution of myoactivity detected between the nerves was similar for the two bioassays in that in both the heart and the oviduct the DN8 extract contained the greatest concentration of myostimulatory factor. The terminal ganglia extract was also myostimulatory in both bioassays although to a lesser extent. Unlike the larval heart, the oviduct showed no response to the VN7, VN8 and TN8 extracts.

Terminal nerves from sexed pharate adults were dissected individually as in previous experiments. Male and female sets of terminal nerves were processed and applied separately to the larval heart bioassay and oviduct bioassays. The results are shown in graph form in Fig. 4.13 for the larval heart bioassay. Examples of chart recorder printouts of the responses of the larval heart are shown in Fig. 4.11 and Fig. 4.12.

All the terminal nerve samples from the male insects were less cardioactive than the female with the exception of the terminal ganglia extracts. The response to the DN8 extracts showed a 200.0% increase in the female and, in the male, a 162.5% increase. The female VN7 extract with a 100.0% increase was four times as cardioactive as the male with a 25.0% raise in heartrate. The VN8 extract from male insects gave a 25.0% rate increase and the female extract gave a 62.5% increase. The male TN8 extract was a third as active as the female with 25.0% and 87.5% increases respectively. The PN extract was not cardioactive in either sex. The female terminal ganglia extract caused a 75.0% increase whilst the male extract was less active with a 50.0% raise in heartrate.

In the oviduct, the female DN8 extract caused a 150.0% increase in contraction rate and the male a 50.0% increase. The response is shown in Fig. 4.14. The

terminal ganglia extracts from the two sexes were of the same myoactivity, with a 100.0% increase over the basal contraction rate. No other extracts were myoactive in the oviduct.

In further experiments, matching pairs of terminal nerves from 20 female pharate adults were pooled, lysed and fractioned by HPLC using a C-4 (Hypersil) column. A factor cardioactive in the larval heart eluted at 25-27% acetonitrile/0.1% TFA from the terminal ganglia, VN7, DN8, VN8 and TN8 extracts. Chart recorder traces of the response of the larval heart to fractions of the terminal nerves are shown in Fig. 4.15. The results are represented graphically in Fig. 4.16. No fraction was found to be myoactive in the oviduct at a concentration of one insect equivalent. Although this lack of response may be due to losses of myoactive material incurred during HPLC processing, this would seem unlikely as levels of cardioactive response of the larval heart were similar for both HPLC fractionated and crude samples. The oviduct bioassay has been shown to be relatively insensitive in comparison to the larval heart bioassay (see Chapters II and III) and preparations are more variable in detecting low doses. The limited amount of test sample available may have reduced the concentrations to levels undetectable by this bioassay and also restricted the number of applications which could be made.

When taken together the fractions obtained from DN8 nerves contained the highest levels of cardioactive factor. The fraction at 26% from the DN8 nerves was the most cardiostimulatory with an increase of 242.9% over the base rate. The 25% fraction caused a 50.0% increase and the 27% fraction a 100.0% increase. The fractions from the terminal ganglia extract were next most cardioactive with the fraction at 26% causing a 128.6% increase, the 27% a 42.9% increase and the 25% a 50.0% increase. The VN7 and VN8 extracts contained similar amounts of

cardioactivity to each other but in total the fractions were approximately a third as active as those of DN8. The TN8 extracts were approximately a fifth as cardioactive as DN8. PN extracts were not cardioactive. The relative distribution of cardioactivity between the fractions of the nerve extracts was not identical although for each of the cardioactive extracts tested the 26% fraction contained proportionally the greatest concentration of the active factor.

Summary

The abdominal nerve cord of both larval and adult *Manduca sexta* contains a peptide or set of peptides which are myoactive in the isolated oviduct of this insect. The nerves exiting the terminal ganglia also contain peptides myoactive in the oviduct and the different sets of nerves vary in the amount of myoactive factor detected in extracts with the activities in descending order: DN8 > VN7 = VN8 > TN8 > PN as measured by both the oviduct and larval heart bioassays. The terminal nerves of females contained approximately 1.8 times as much cardioactivity as those of the male. The DN8 extract from females is three times as myoactive in the oviduct as that from males but in the larval heart the DN8 extracts from males and females have a more similar cardioactivity. A set of fractions were separated from extracts of abdominal nerve cords by HPLC and the same fractions found to be active in the oviduct were also active in the larval heart. Separation of the terminal nerves revealed that the factor cardioactive in the larval heart eluted in the same set of fractions from all the terminal nerves.

4.3.2 Visualisation of the Oviducal Nerves

Methylene Blue Staining

Thorn and Truman (1989) described the innervation of the female reproductive tract in *Manduca sexta* and found it to be nearly identical to that of the moth *Pectinophora gossypiella* (Cook *et al.* 1980).

The stain methylene blue can be used to highlight nerves and was used in the staining of partially dissected pharate and emerged adult moths. The stain enabled the terminal nerves to be identified and traced, revealing the gross pattern of innervation and allowing the identification of the target organs and tissues of each nerve.

In adult *Manduca* five pairs of nerves extend from the caudal end of the fused terminal ganglia. These are; ventral nerve 7 (VN7), dorsal nerve 8 (DN8), ventral nerve 8 (VN8), terminal nerve 8 (TN8) and proctodeal nerve (PN). The pattern of the terminal nerves is shown in Fig.4.1.

Figure 4.2 is a diagrammatic representation of the female reproductive tract drawn from ten methylene blue stained preparations and showing the gross pattern of innervation of the different regions.

The nerve VN7 bifurcates and one branch extends to innervate the 8th abdominal spiracle closer muscle and intersegmental body wall muscles. At the lateral oviduct the nerve divides again with one branch connecting with the ventral surface of the lateral oviducts and ramifying extensively across the surface. The

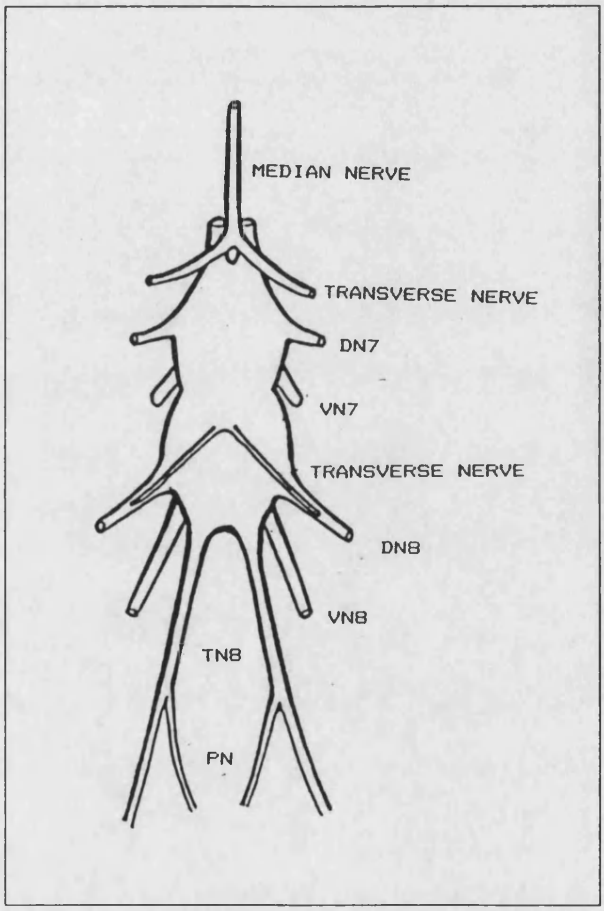

Figure 4.1: Diagram showing the nerves exiting the fused terminal ganglia in the adult *Manduca*.

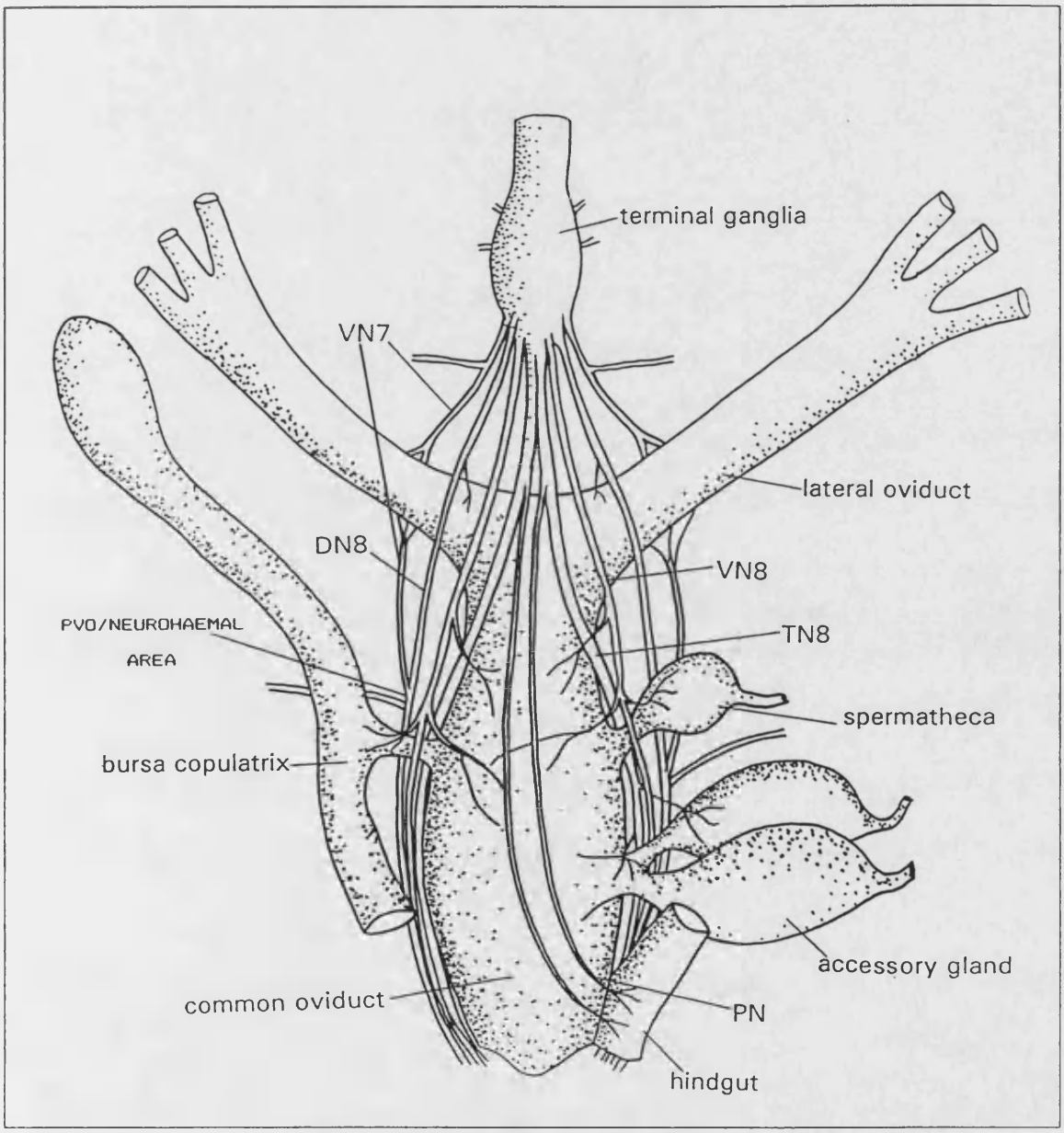

Figure 4.2: Diagram showing the gross innervation of the female reproductive tract from the terminal ganglia.

other passes ventrally under the lateral oviduct and connects with DN8. The DN8 nerves pass dorsally over the lateral oviducts with a minor branch leading to the dorsal surface of the lateral oviduct. DN8 extends past the anterior of the common oviduct without direct innervation of the muscles and reaches a swelling- a neurohaemal area (PVO, perivisceral organ) and connects with the branch of VN7 which extends past the lateral oviduct. Three nerves then exit the PVO with two extending to the terminal spiracle closer muscle, and associated somatic musculature and the third continuing to the ovipositional musculature. The VN8 nerve passes dorsally over the lateral oviduct/junctional area and innervates both the dorsal and ventral surfaces of the anterior common oviduct. The nerves continue to the ovipositional musculature with both nerves of the pair innervating the posterior common oviduct, the bursa copulatrix and the spermatheca. A fine branch of VN8 can be traced up the length of the bursa copulatrix. The spermatheca is in comparison to other tissues rich in both nerves and tracheoles. The TN8 nerve passes dorsally over the junctional region of the lateral oviducts and innervates the accessory glands with branches bifurcating to connect separately to the gland and duct regions. Fine nerves cover the surface of the glands. The PN nerve is a branch of the terminal nerve, and leads directly with no branching to innervate the hindgut (Reinecke *et al.* 1973).

Cobalt Nerve Fills

Cobalt back and forward filling of individual nerves allows detailed investigation of the nerve-target tissue interface and of the cell bodies supplying the nerves in the ganglia.

Limited success was achieved in backfilling into the ganglia in this study. Analysis

of all the backfilled preparations was hampered because of excessive colouration of the specimens. Forward filling of DN8 did not extend beyond the PVO in any preparation. Forward filling of VN7 was successful and showed extensive bifurcation of this nerve over the surface of the lateral oviducts with little or no innervation of the junctional region as shown in Fig. 4.3. The complexity of the filling pattern and the problem of obtaining sufficient depth of field precluded photographic representation of the results.

Immunohistochemistry – Anti-CCAP Staining

Four wholemounts of the abdominal nerve cord and female reproductive system were immunohistochemically stained using antiserum directed against synthetic CCAP (antiserum 2TB, Dircksen and Keller 1988).

Neurons and nerve fibres positive to anti-CCAP antiserum were detected throughout the abdominal nerve cord. In all the ganglia, the transverse nerves were heavily stained. In the nerves extending to the reproductive system, the positive staining appeared to be of neurohaemal release sites along specific nerves. Figs. 4.17, 4.18 and 4.19 are photographs showing the staining pattern with this antisera.

In each of the abdominal ganglia 3 – 5, nine immunopositive cell bodies were detected these were; a pair of lateral neurons anterior to the root of the dorsal nerves, four lateral neurons, a pair of midline neurons and a single midline cell. Only very weak staining of neurons in the terminal ganglia was obtained and identification was not possible.

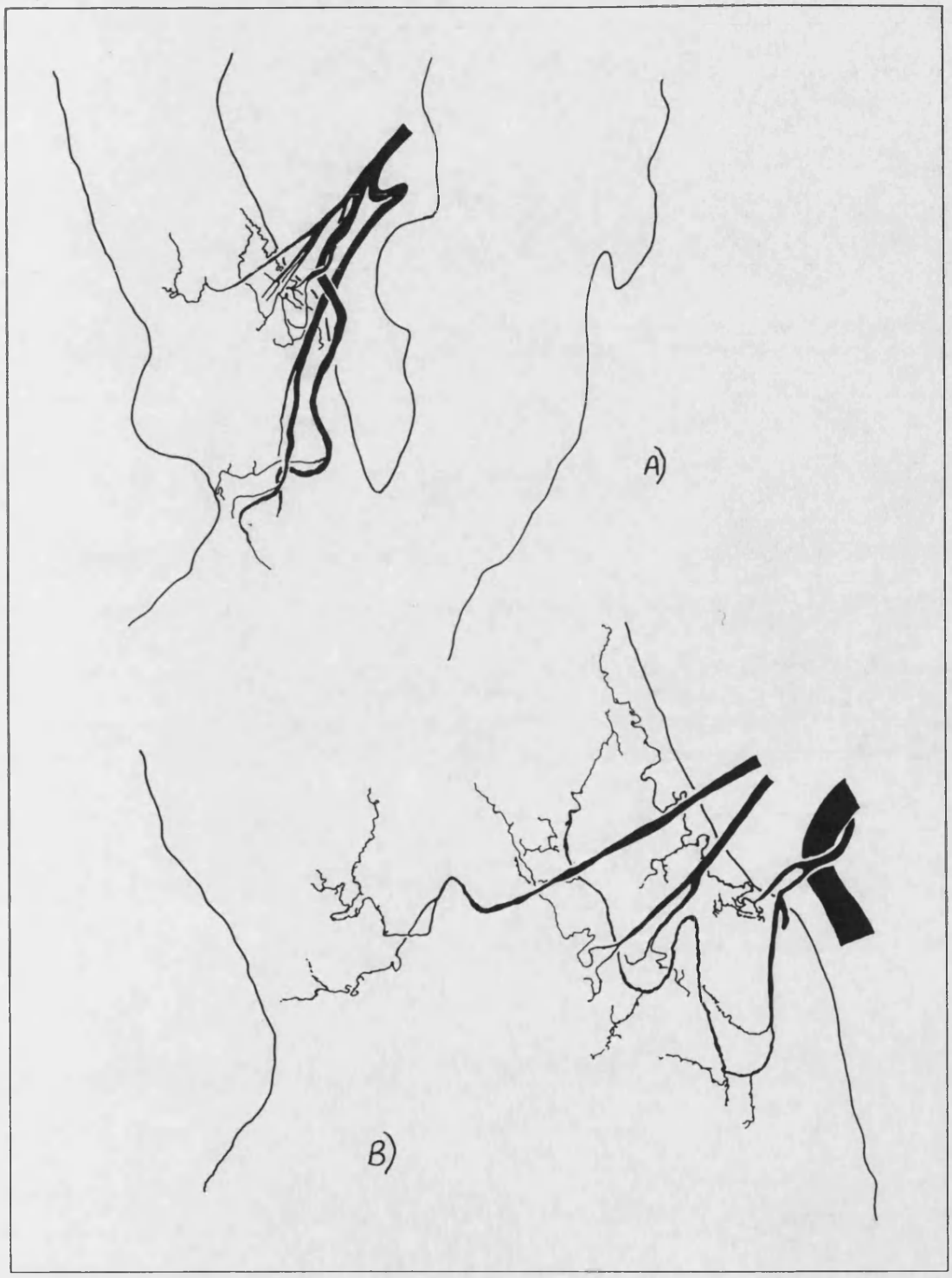

Figure 4.3: Drawing of a forward fill of VN7

A) low power and B) high power magnification showing the paired lateral oviducts, the joining region, the anterior of the common oviduct and VN7 dividing and ramifying over the surface of one lateral oviduct.

In all four preparations the DN8 nerves extending from the terminal ganglia were positively stained. In two of the preparations some of VN7 and areas of TN8 were stained but more weakly. Staining appeared to show neurohaemal areas along the length of the nerves with blebs on the nerve surface rather than fibres running along the nerve. The staining in DN8 was along the entire length of the nerve however the associated PVO was not retained in any preparation. In some preparations the VN7 nerve between the lateral oviducts and the join with DN8 was retained and this showed anti-CCAP immunoreactivity.

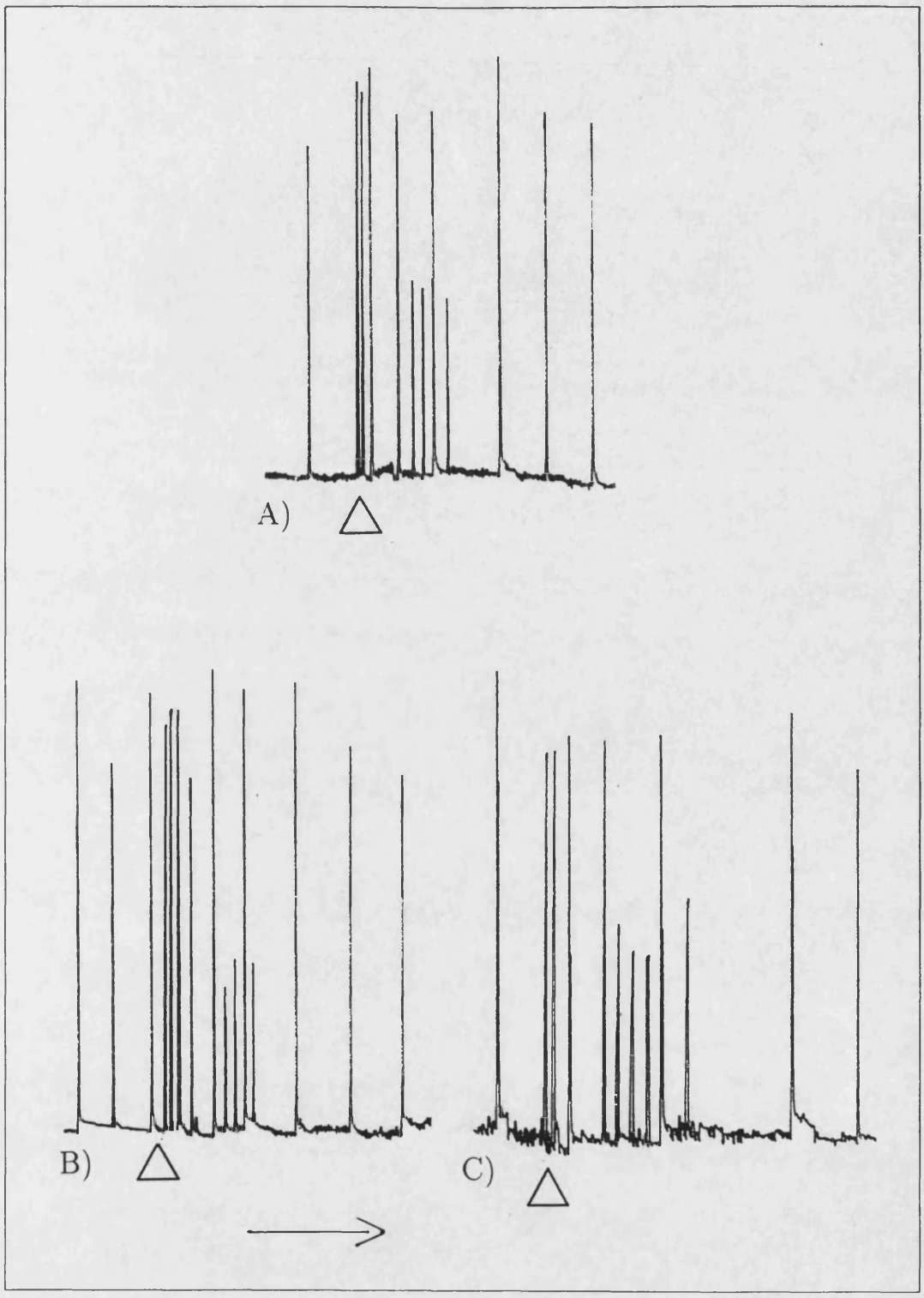

Figure 4.4: Figure showing the response of an isolated pharate adult oviduct to extracts of abdominal nerve cord.

Extracts applied at a concentration of 0.2 insect equivalents, A) – larvae at 1 day prior to wandering, B) – pharate adult females, C) – pharate adult males. Chart speed of 0.25 cm/minute. Horizontal arrow shows direction of recording.

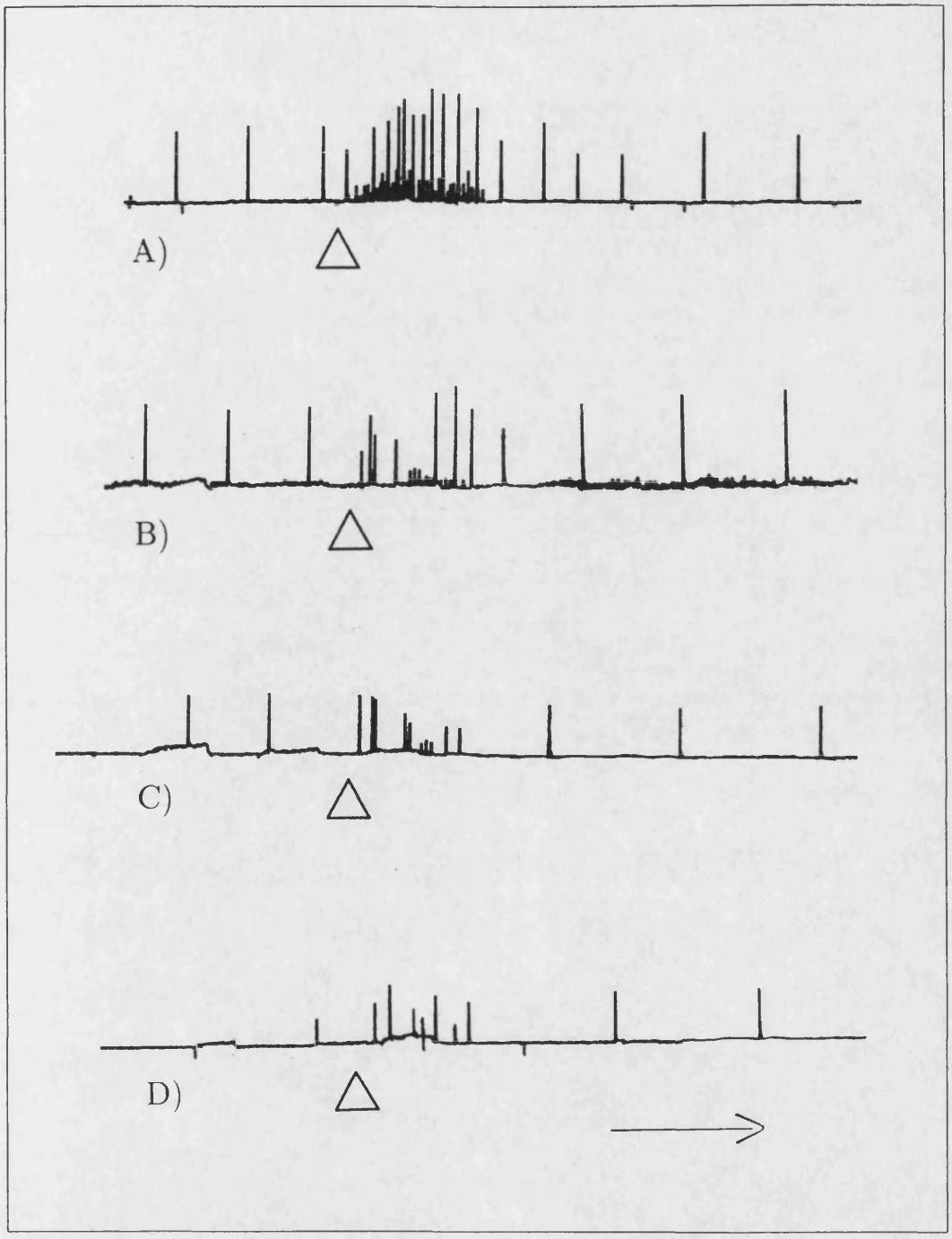

Figure 4.5: Figure showing the response of a pharate adult oviduct to extracts of abdominal nerve cord from pharate adults.
A) – 0.5 insect equivalents, B) – 0.2 insect equivalents, C) – 0.1 insect equivalents, D) – 0.05 insect equivalents. The chart speed is 0.25 cm/minute.

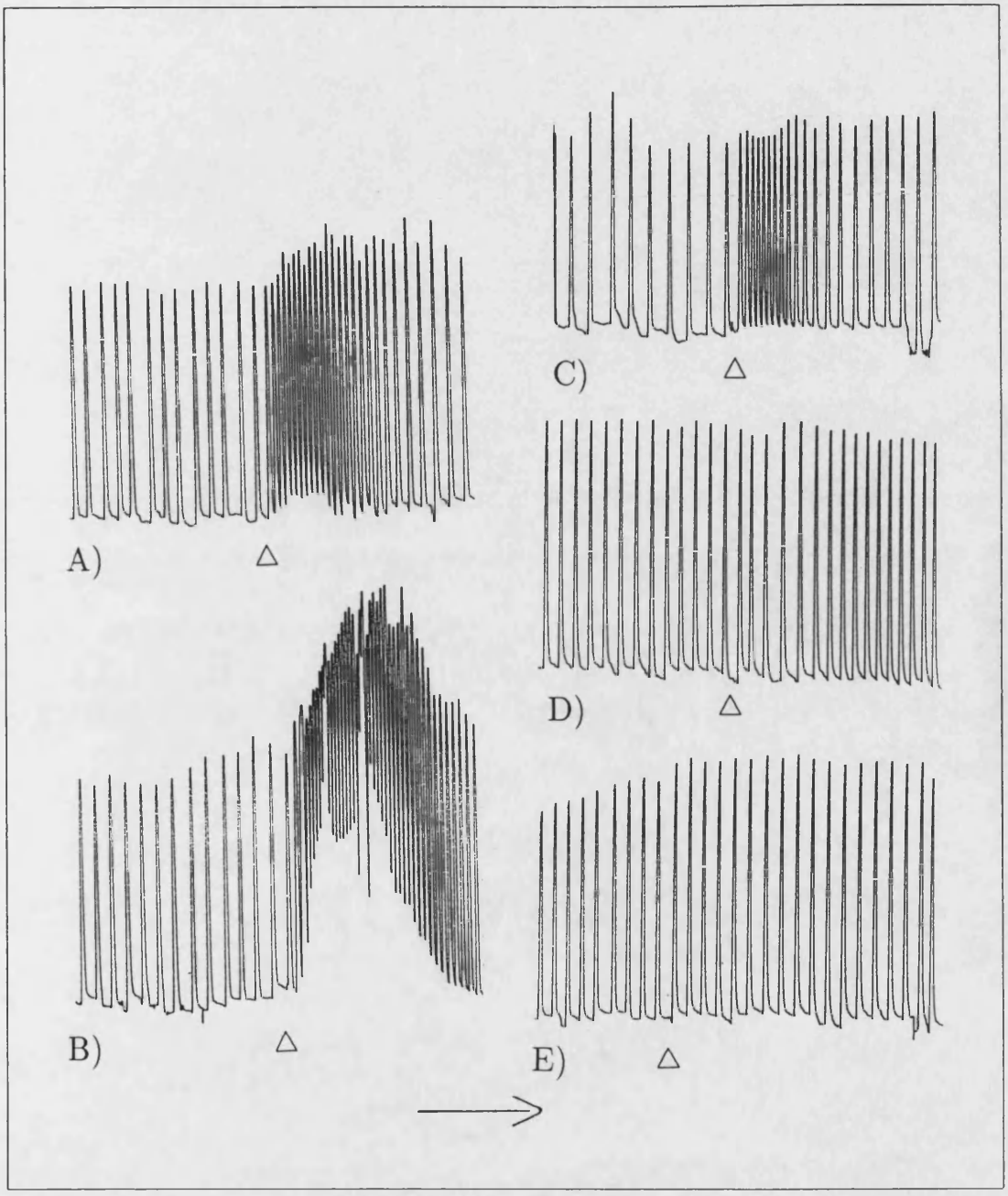

Figure 4.6: Figure showing the response of the semi-isolated larval heart to HPLC fractions of adult abdominal nerve cord extract.
Extracts separated using a C-4 column. Each application was 0.25 insect equivalents. A) – fraction 25, B) – fraction 26, C) – fraction 27, D) –fraction 28, E) – fraction 29.

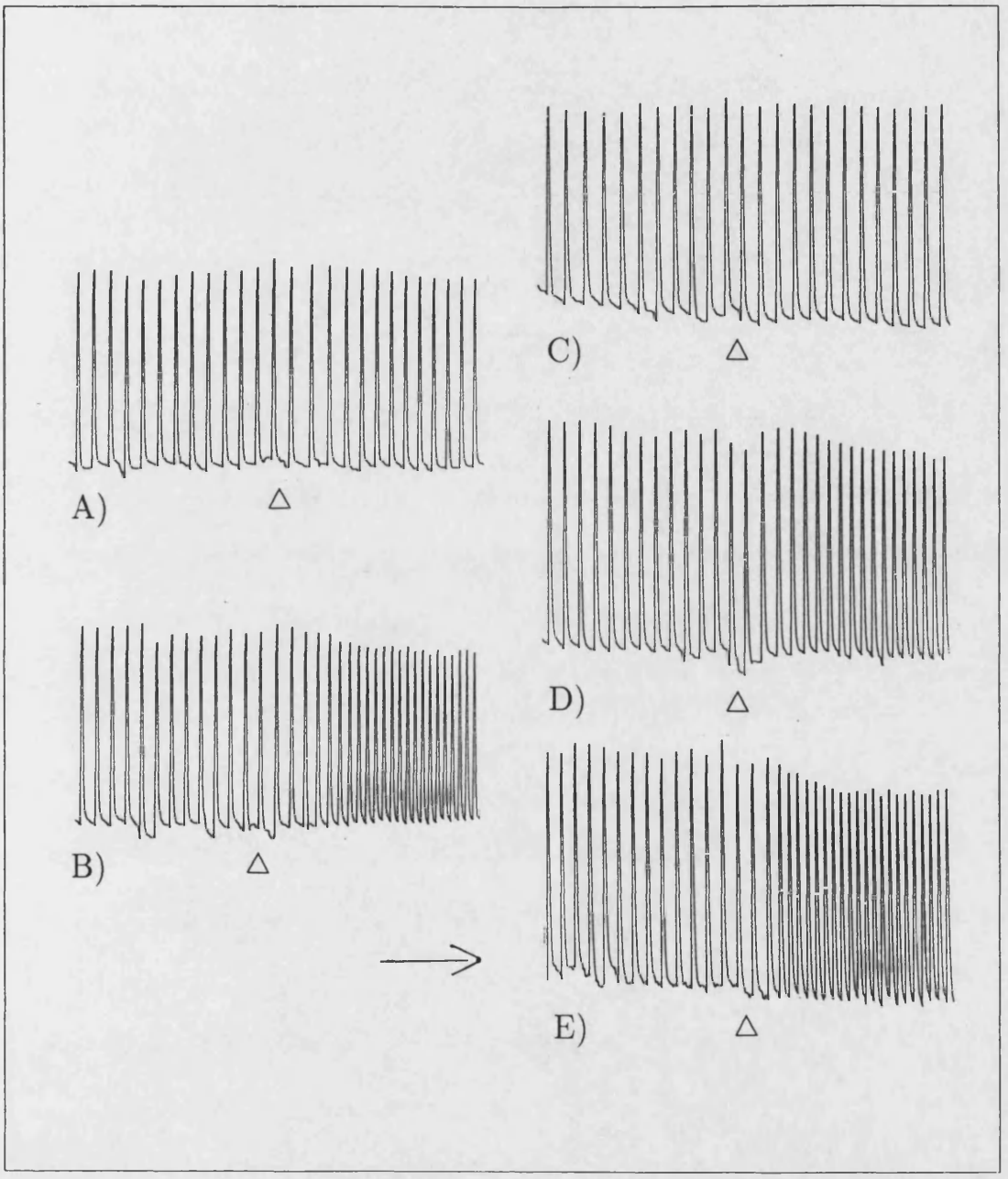

Figure 4.7: Figure showing the response of the semi-isolated adult heart to HPLC fractions of adult abdominal nerve cord extract.
Extracts separated using a C-4 column. Each application was 0.25 insect equivalents. A) – fraction 25, B) – fraction 26, C) – fraction 27, D) – fraction 28, E) – fraction 29.

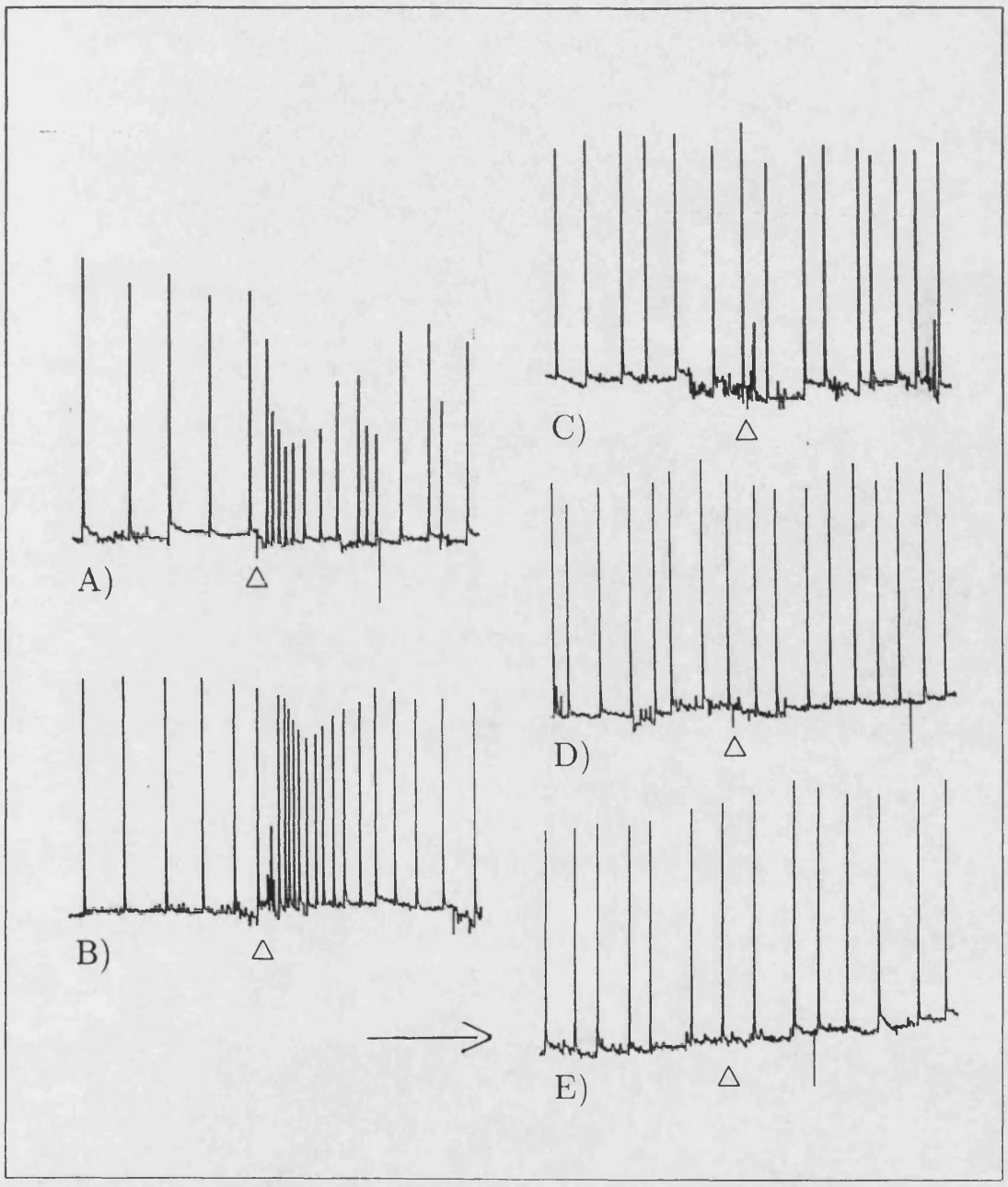

Figure 4.8: Figure showing the response of the isolated pharate adult oviduct to HPLC fractions of pharate adult abdominal nerve cord extract.
Extracts separated using a C-4 column. Each application was 0.5 insect equivalents. A) – fraction 25, B) – fraction 26, C) – fraction 27, D) – fraction 28, E) – fraction 29.

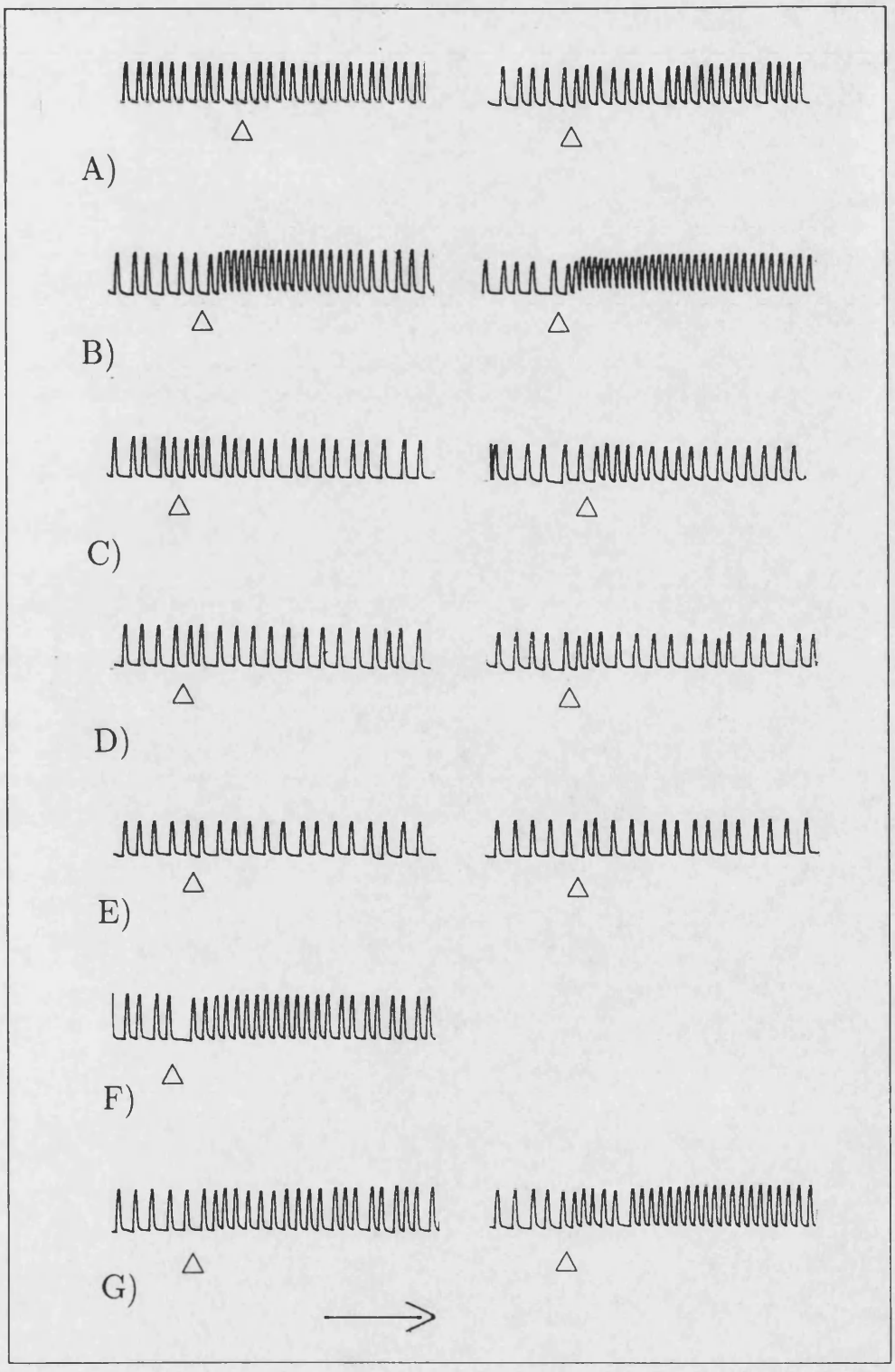

Figure 4.9: Figure showing the response of the larval heart to extracts of the terminal nerves of pharate adults.
Applications were of 0.25 and 0.5 insect equivalents. A) VN7, B) DN8, C) VN8, D) TN8, E) PN, F) terminal ganglia, G) penultimate ganglion.

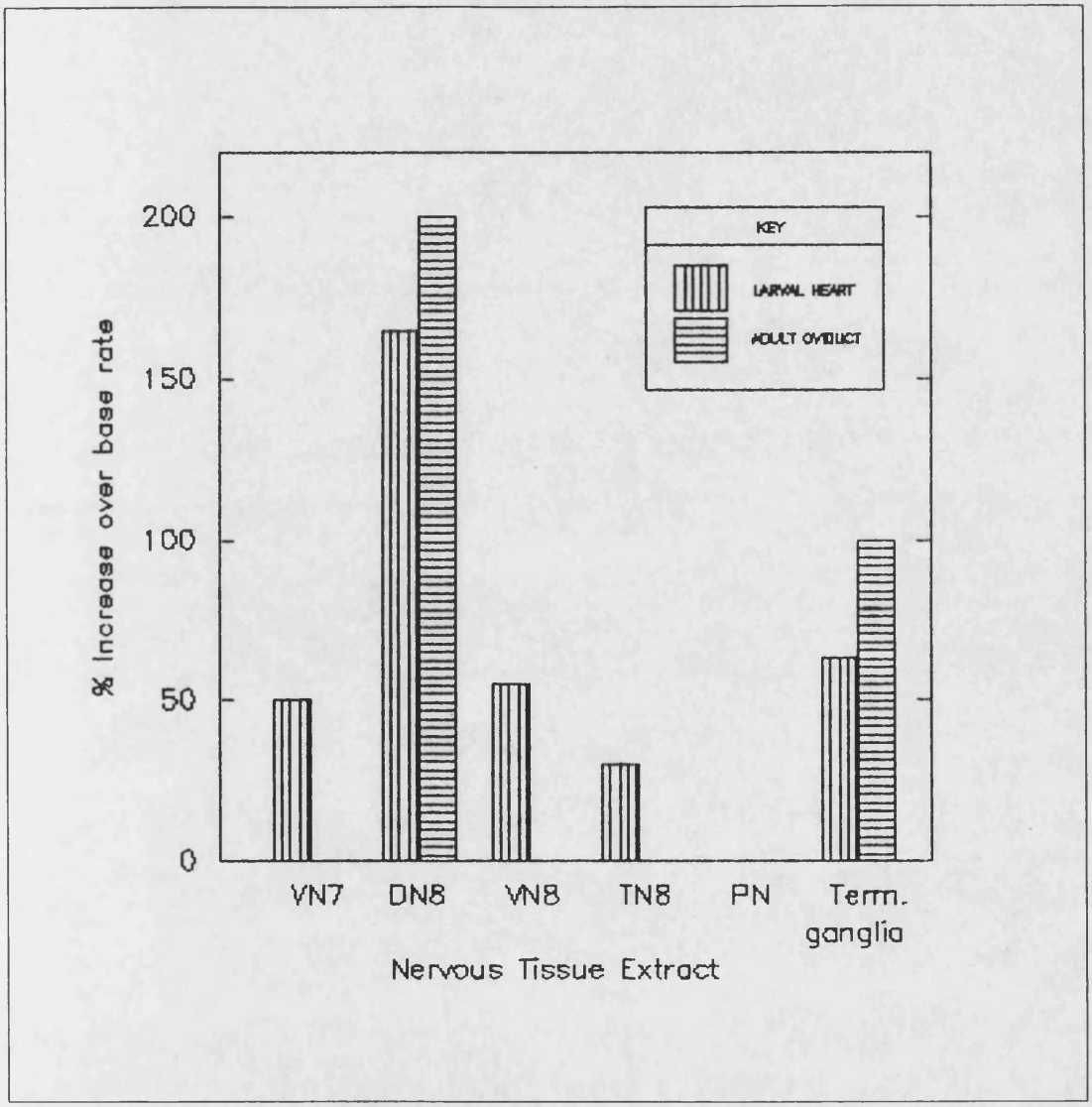

Figure 4.10: Graph showing the response of the larval heart and the adult oviduct to extracts of the terminal nerves of pharate adults.
Larval heart applications 0.25 insect equivalents, oviduct 0.5 insect equivalents.

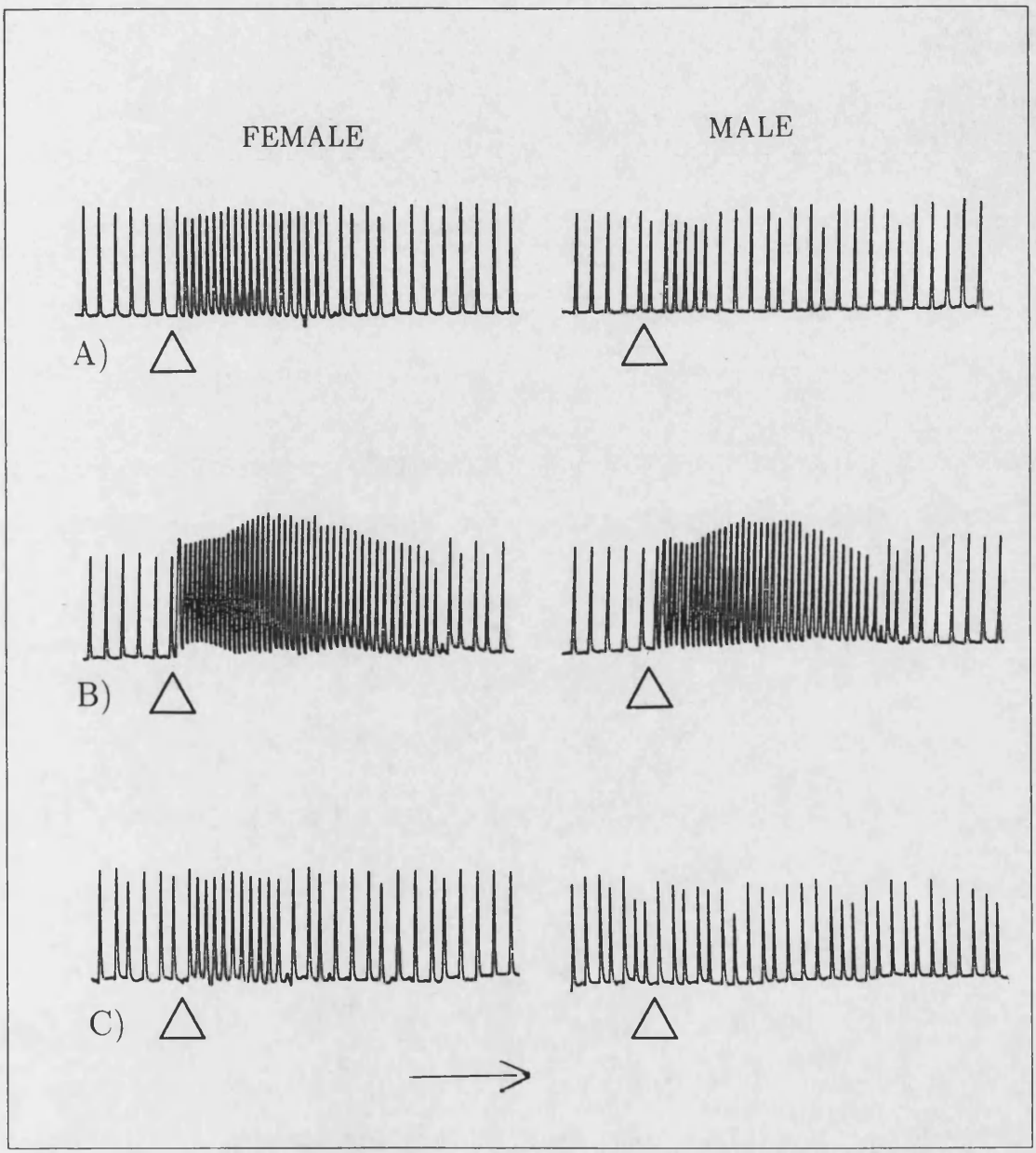

Figure 4.11: Figure showing the response of the larval heart to extracts of the terminal nerves of female and male pharate adults.
Applications were of 0.5 insect equivalents. A) VN7, B) DN8 and C) VN8.

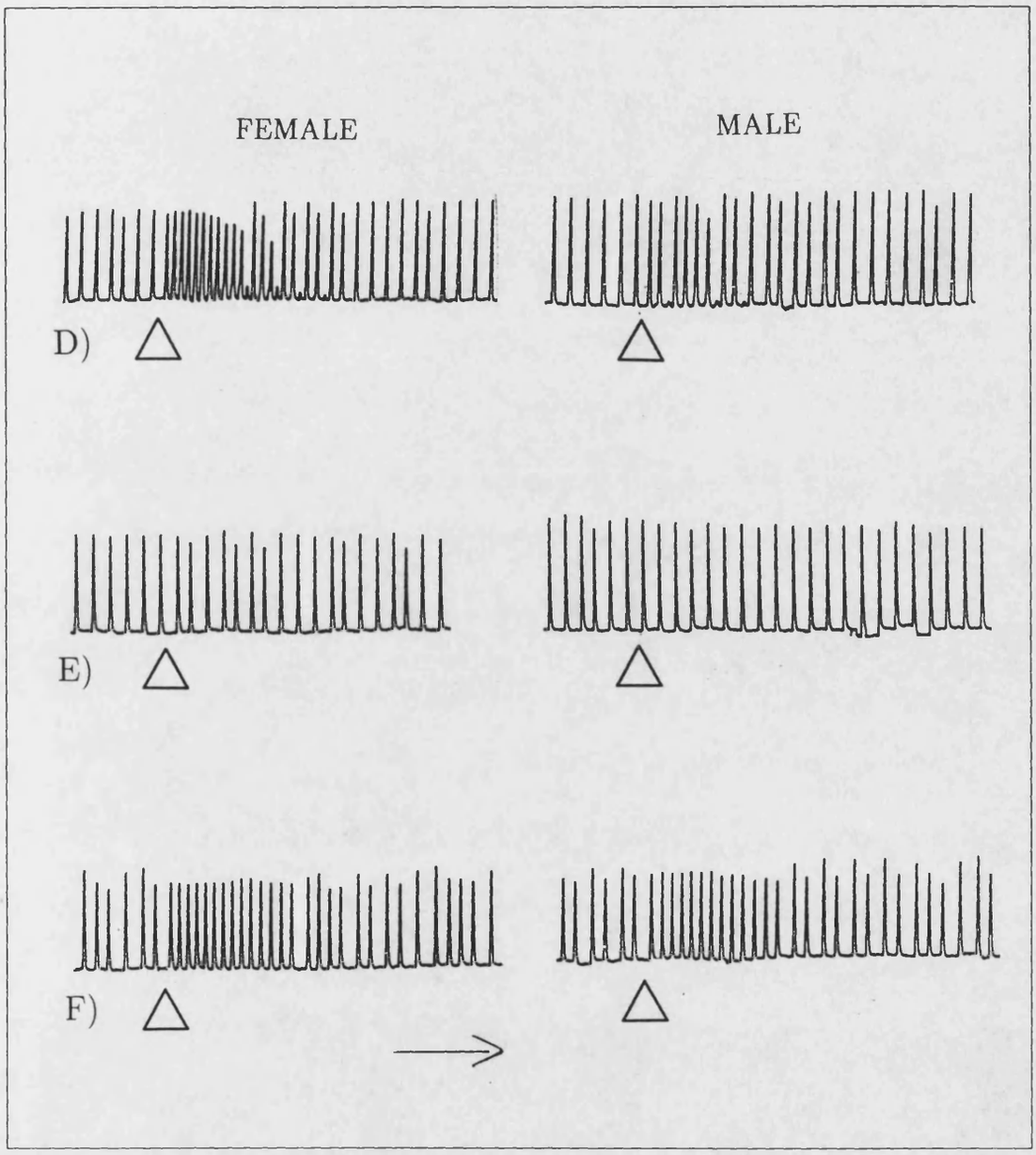

Figure 4.12: Figure showing the response of the larval heart to extracts of the terminal nerves of female and male pharate adults.
continuation of previous figure. D) TN8, E) PN and F) terminal ganglia.

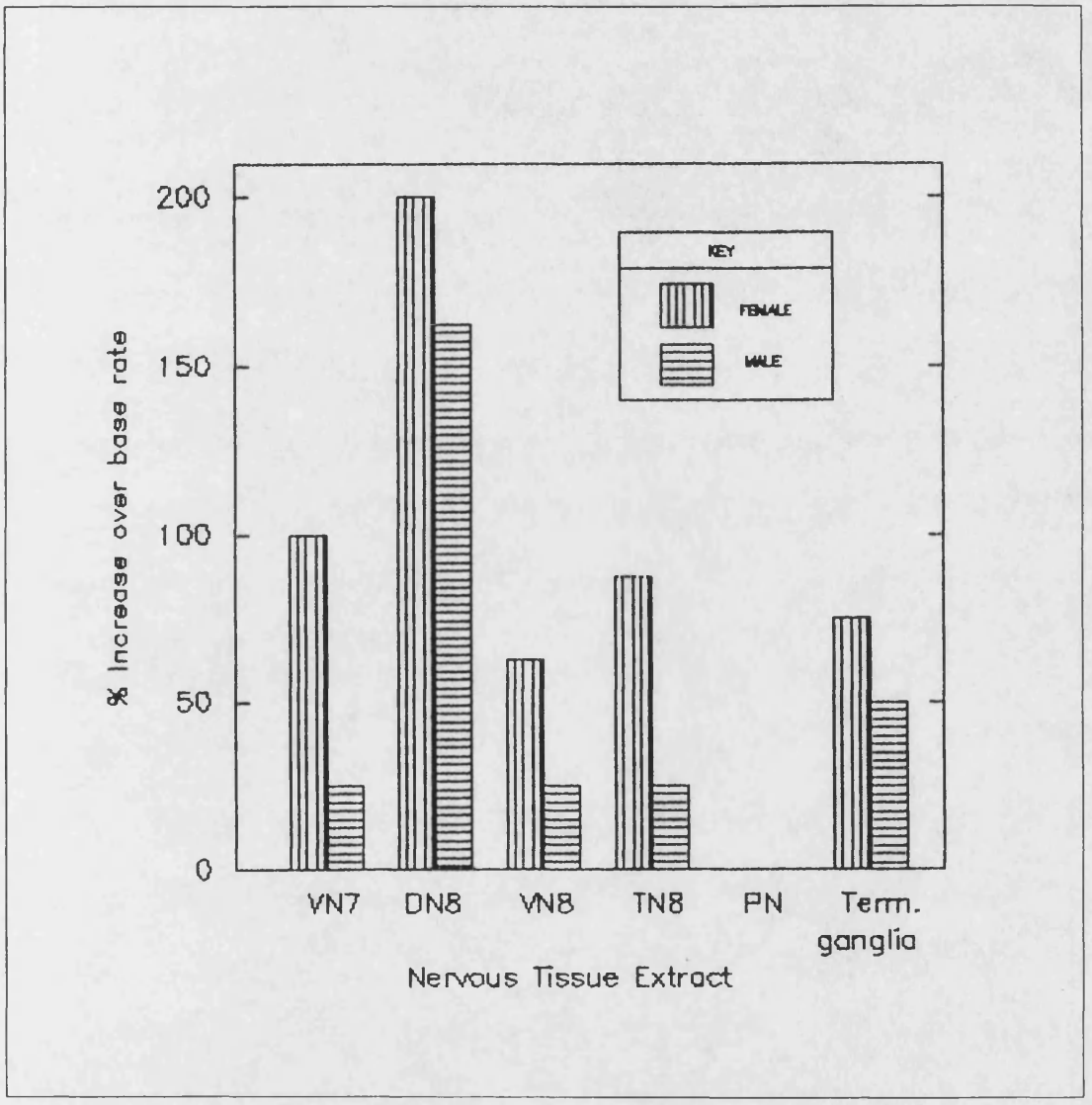

Figure 4.13: Graph showing the response of the larval heart to extracts of the terminal nerves of male and female adult moths.
Conditions as for previous figure.

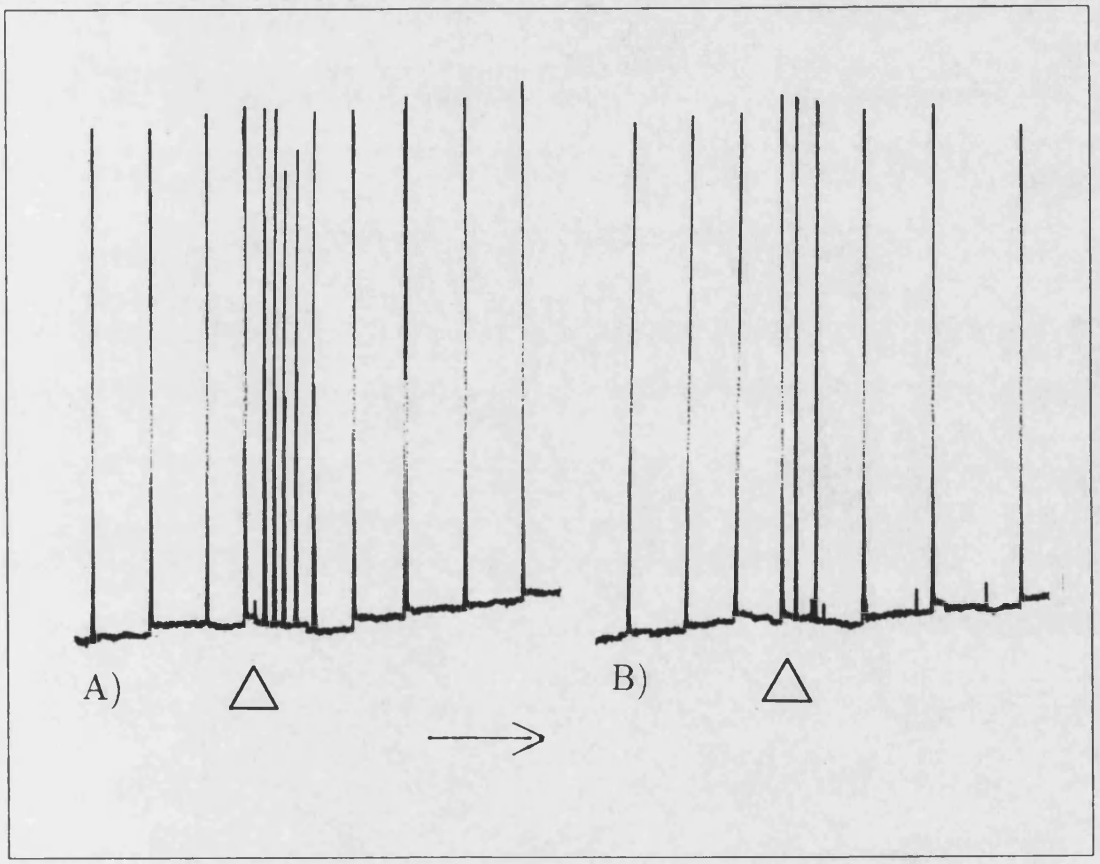

Figure 4.14: Figure showing the response of the oviduct to extracts from the DN8 nerves.
A) female and B) male pharate adults. The chart recorder speed was 0.25 cm/minute. Extracts were applied in 100μl at 0.5 insect equivalents.

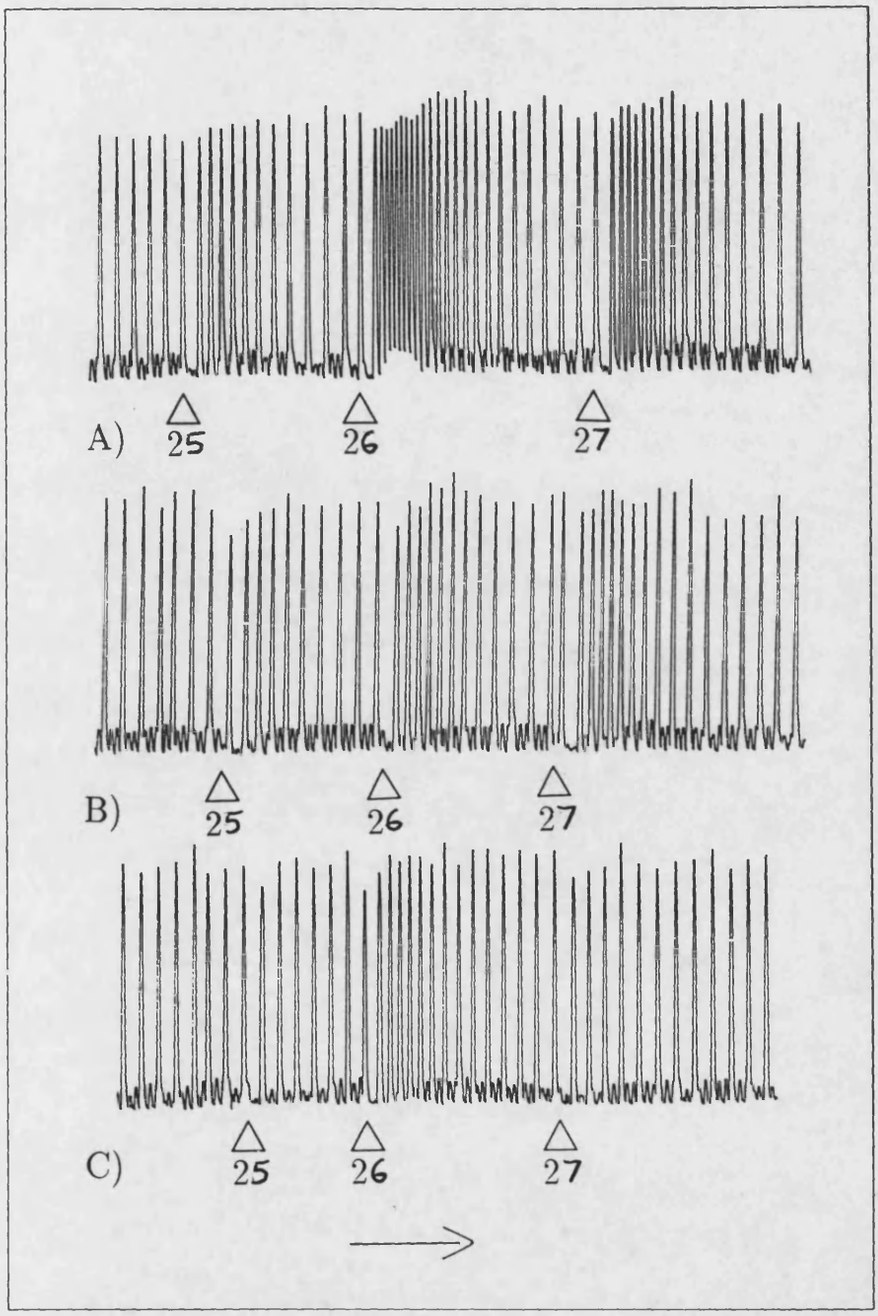

Figure 4.15: Figure showing the response of the larval heart to HPLC fractions of terminal nerve extracts.
Data for VN7 is not shown. Applications were made at a concentration of 1 insect equivalent. A) DN8, B) VN8, C) TN8. Chart speed was 2.5 cm/minute.

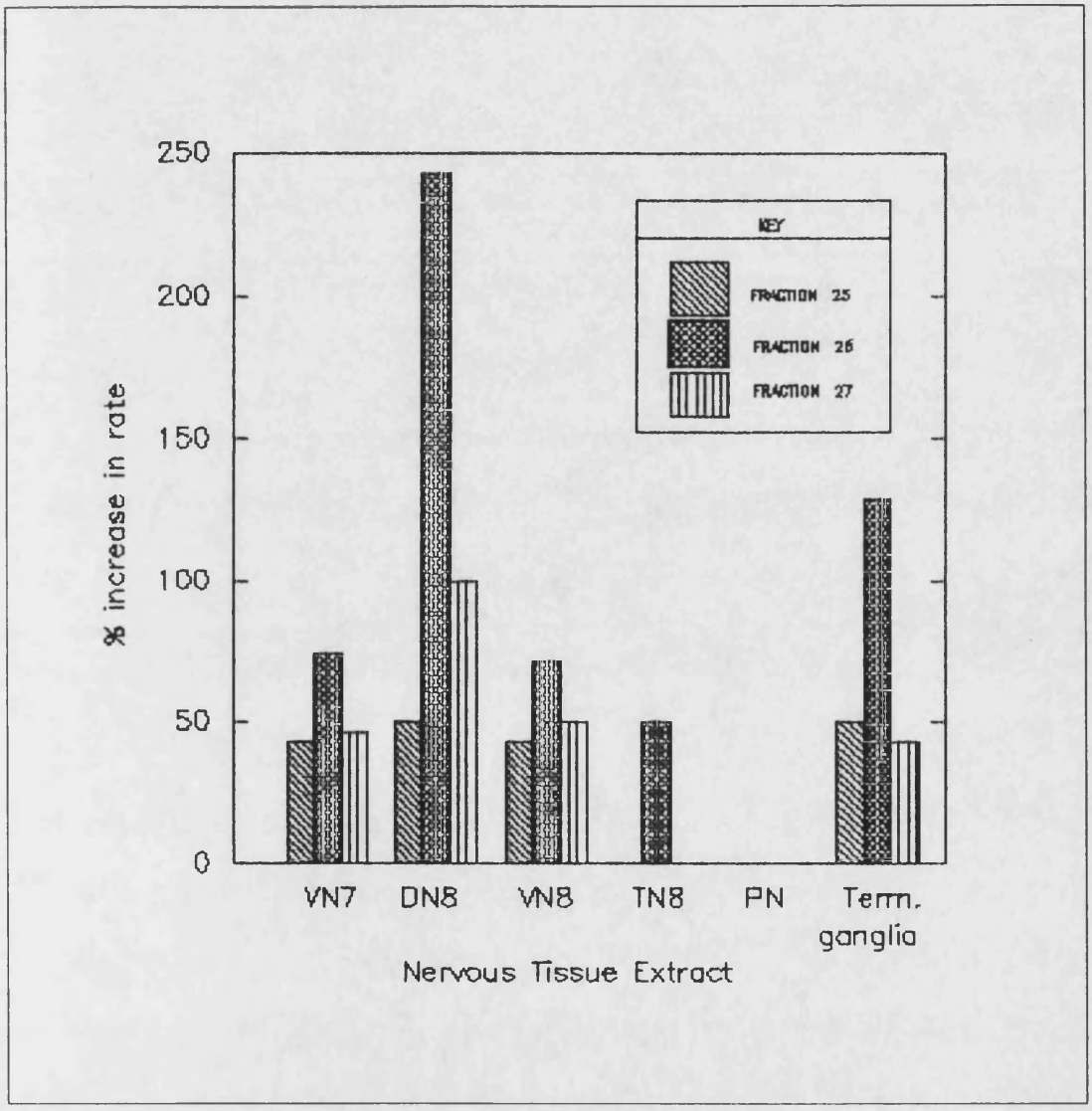

Figure 4.16: Graph showing the response of the larval heart to HPLC fractions of the terminal nerves.
Conditions as for previous figure.

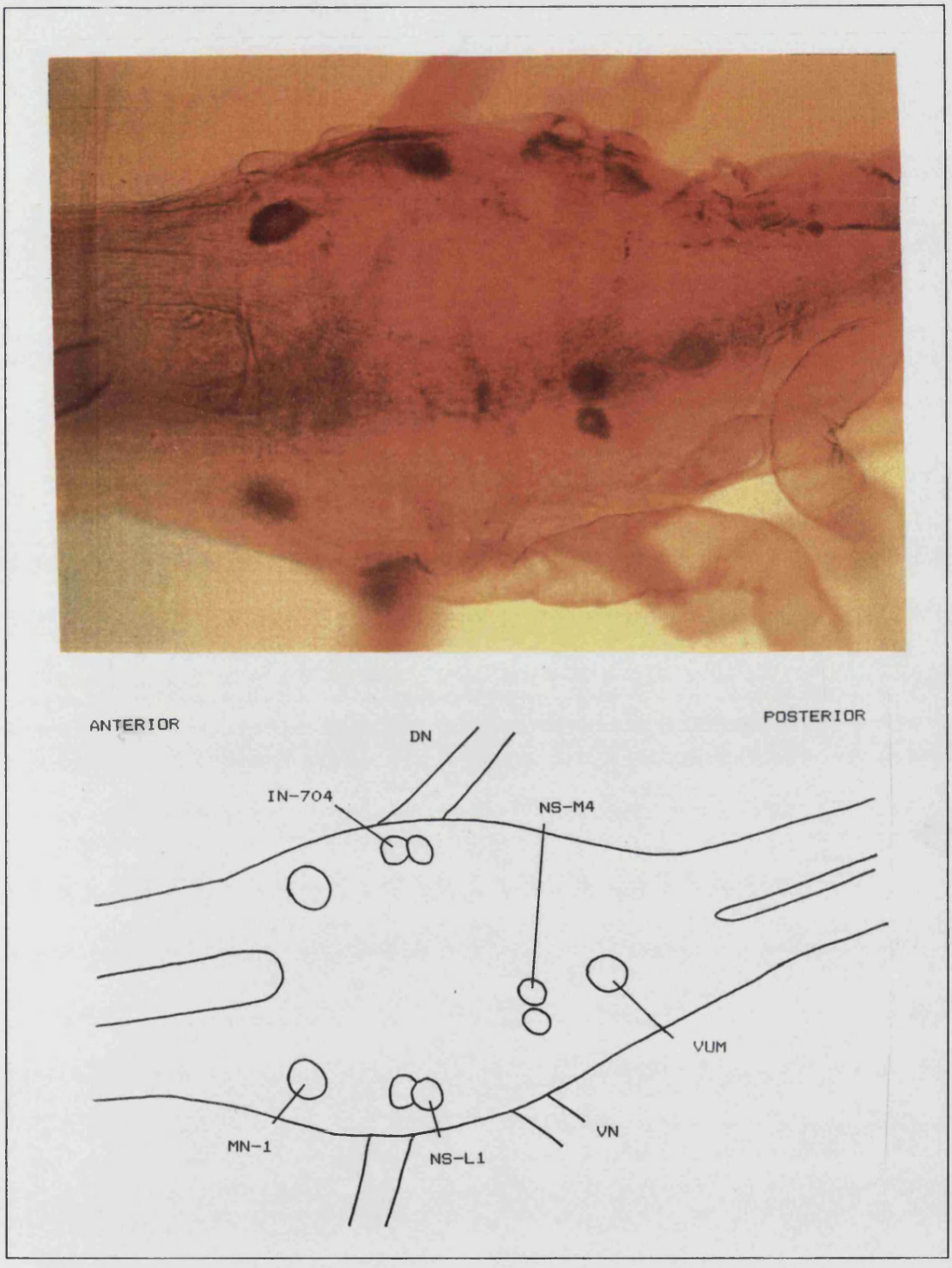

Figure 4.17: A) Photograph of whole mount showing anti-CCAP immunoreactive neurons in the abdominal ganglia 5, B) Diagramatic representation of anti-CCAP neurons detected in the unfused abdominal ganglia.

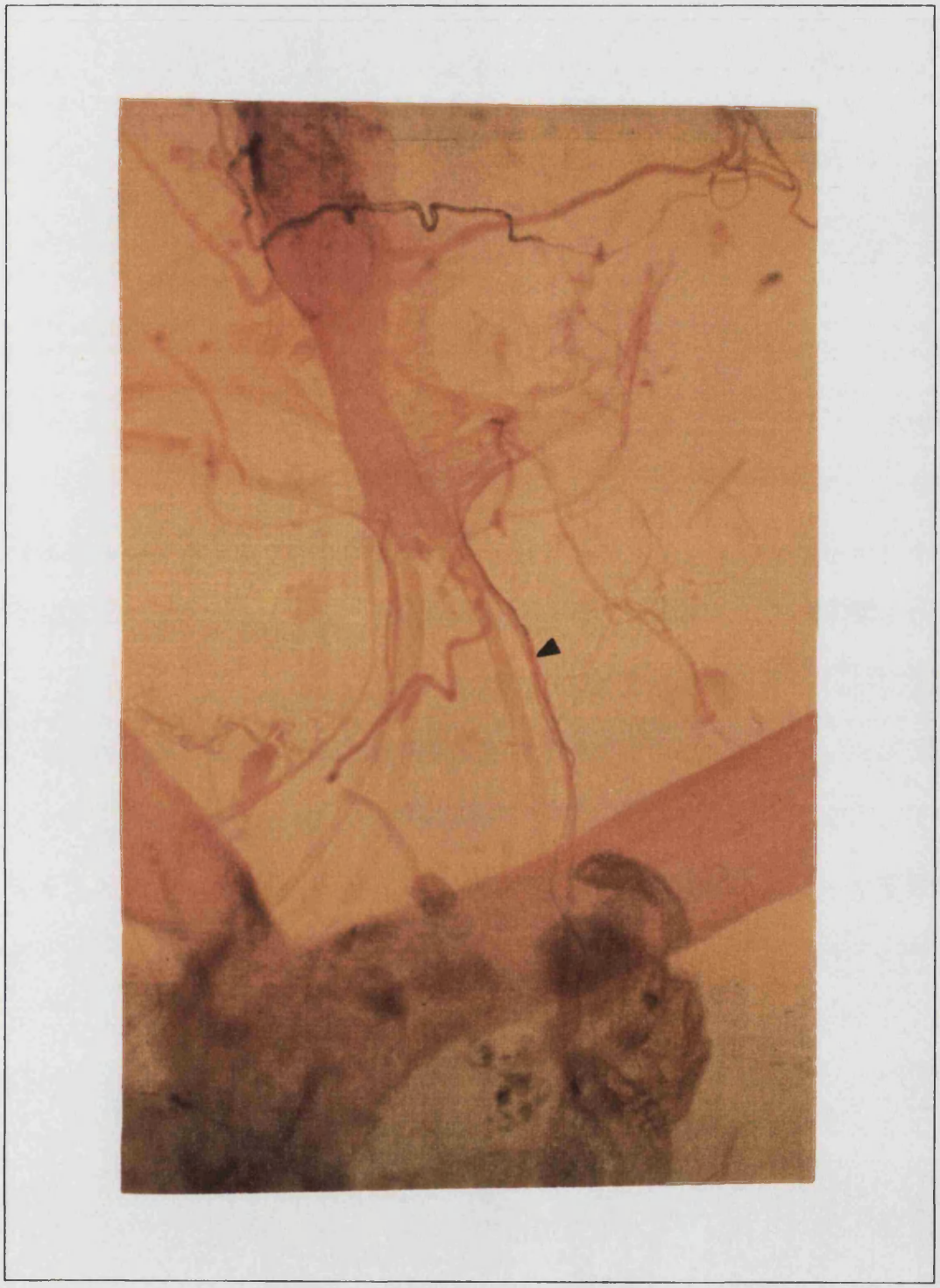

Figure 4.18: Photograph of the lateral oviducts and abdominal nerve cord of whole mount showing the nerve DN8 stained by anti-CCAP antibody.

Figure 4.19: A) Enlarged view of DN8 exiting terminal ganglia and passing dorsally over lateral oviduct. B) Photograph showing anti-CCAP immunoreactivity in DN8 in neurohaemal-type areas.

4.4 Discussion

The experiments described in this chapter were directed towards obtaining a better understanding of the neurohormonal regulation of the *Manduca* oviduct. The novel oviduct bioassay detailed in Chapter III of this study has enabled an investigation into the endogenous oviduct myostimulatory factors. Results presented here show that the myogenic contractions of the oviduct in this insect can be modulated *in vitro* by factors present in the abdominal nerve cord. These factors occur in both the larval and adult abdominal nerve cords and the concentration in each nerve cord does not vary with age or sex. Application of crude nerve cord extract stimulated a biphasic response with the initial phase of contractions slowing and then being followed by a second set of contractions. This pattern of response may indicate the presence of two stimulatory substances, one acting immediately and the other with more delayed effects or, alternatively, the presence of an inhibitory substance in addition to the stimulatory factor.

The HPLC analysis of adult nerve cords demonstrated that the same fractions which are cardioactive in the larval heart are myostimulatory in the oviduct and that this fraction is one of the two which are cardioactive in the adult heart. Analysis of the larval nerve cords showed that the oviduct responded to the same fraction as both the adult and larval hearts. The larval nerve cord is known to contain two sets of peptides cardioactive in the larval heart (Platt and Reynolds 1985) however only one of these sets was detected in the larval heart bioassay in this study. The reason for this is not known.

The use of the *Manduca* oviduct bioassay in conjunction with the adult and larval heart bioassays allows the likely identity of the oviduct stimulating peptide in the *Manduca* nerve cord to be deduced. The abdominal nerve cord of *Manduca* is

known to contain two sets of cardioacceleratory peptides. In the adult these peptides have been termed the CAP1s and CAP2s and CAF1s and CAF2s in the larva. The CAP2s are known to be very similar or identical to the CAF2s but the CAP1s are not the same as the CAF1s (Tublitz *et al.* 1992). The CAP1s are not cardioactive in the larval heart and the CAF1s are not cardioactive in the adult heart.

The results from this study show that the larval heart responds to the same fractions from the adult nerve cord as the oviduct and that the adult heart responds to the same fractions from the larval nerve cord as the oviduct. The oviduct myoactive fractions from both adults and larvae elute at the same time under similar separation conditions. This confirms that members of the CAP2 set of peptides are involved in oviduct motility *in vivo*.

One of this set of peptides has been isolated and sequenced and is known to be identical to crustacean cardioactive peptide (CCAP) (Cheung *et al.* 1992), a novel cyclic nonapeptide first isolated from the shore crab, *Carcinus maenas* (Stangier *et al.* 1987). This peptide has been shown to be a potent stimulator of oviducal contractions in *Manduca sexta* (this study, Chapter III). The link between CCAP and myoactive factors in the nerve cord is investigated in greater detail in Chapter V. Synthetic CCAP was not tested under the HPLC conditions described in this chapter due to the likelihood of column contamination nullifying further results. Detection of CCAP immunoreactivity in the nerves extending from the terminal ganglia shows that the peptide is present in both DN8 and VN7. DN8 passes dorsally over the lateral oviduct to connect with a perivisceral organ (PVO) at the junction with VN7. The PVOs have been shown to be the primary neurohaemal release sites for the insect abdominal nerve cord (Raabe 1982). CCAP immunoreactivity (this study and Davies *et al.* 1993) and

CAP bioactivity (Tublitz and Truman 1985a, d) is associated with the PVOs, suggesting that the peptide is released as a circulating neurohormone from these nerves and may act to modulate the contractions of the oviduct by this means. The results from dissection of the terminal nerves show that the oviduct stimulatory factor is present in greatest concentrations in the DN8 nerves. To some extent this result may reflect the method of dissection as the PVOs were included with the DN8 sample rather than the VN7 but supports the evidence from the immunohistochemical study showing that CCAP is present in DN8 nerves.

A description of the pattern of innervation associated with the segmental abdominal ganglia 3 – 5 is given in detail in Chapter I (see also Fig. 1.3). In brief, the transverse nerve arises from the median nerve and carries the PVO. A branch of the ventral nerve (VN) from the preceeding ganglia joins the transverse nerve. The dorsal nerve (DN) links with the distal end of the transverse nerve at the spiracle and the transverse nerve then continues and innervates the alary muscles of the heart. The pattern in the terminal ganglia is different from this in that the ventral nerve (VN7) of the previous fused abdominal neuromere apparently links with the dorsal nerve (DN8) of the next neuromere rather than the transverse nerve. At the joining between DN8 and VN7 is a neurohaemal area likely to be equivalent to the neurohaemal/PVO region of the transverse nerve in other abdominal ganglia. It would seem likely that DN8 and the transverse nerve 8 lie close together and are possibly fused and therefore the DN8 samples used in this study represent both this nerve and the transverse nerve of the same neuromere. This suggestion is further supported by the observation that considerable CCAP immunoreactivity is present in the transverse nerves of ganglia 3 – 5 and DN8 also contains this peptide. The extent of CCAP immunostaining in the distal regions of dorsal nerves from other ganglia is limited (Davies *et al.* 1993) whereas in DN8 anti-CCAP immunoreactivity extends for the length of the nerve that

was retained in the whole mounts.

Cobalt backfills of the nerve DN8 were not successful in this study however Giebultowicz and Truman (1984) made a detailed study of the neurons in the terminal ganglia which have fibres extending along DN8 by the same method. These workers found one ipsilateral, three contralateral and one midline pair of neurons filled from this nerve in adult female *Manduca sexta.* The same pair of midline cells were filled from VN7. Although positive identification is not possible, this pair of midline cells are probably equivalent to the pair of neurosecretory neurons recognised by anti-CCAP antiserum in abdominal neuromere 7 of the terminal ganglia (Davies *et al.* 1993) giving additional support to the evidence presented in this chapter for the presence of CCAP in DN8 and VN7.

The oviducts of insects are both hormonally regulated (Highnam 1962, Okelo 1971 and Giradie and Lafon-Cazal 1972) and innervated (Hartmann and Loher 1974, Thomas 1979 and Cook *et al.* 1980) and are therefore likely to be under additional neural control. The female reproductive tract in *Manduca sexta* is innervated by four sets of nerves and, in addition has been shown in this study (see Chapter III) to possess an intrinsic rhythm of contractile activity which is modulated by a number of neuropeptides. Studies of the regulation of oviducal motility in other insects has led to the suggestion that whilst the myogenic control of contractions and thus egg-laying is regulated by neurohormones, the progression of eggs into the common oviduct is neurally controlled. In the stick insect, *Carausius morosus* experiments involving section of oviducal nerves led to the conclusion that nerves controlled movement of eggs into the common oviduct (Thomas and Mesnier 1973). In the locust, Lange *et al.* (1984) concluded that egg-laying at inappropriate times was prevented by neural inhibition. Stimulation of the oviducal nerves resulted in the movement of eggs back towards the ovaries.

Recordings from these nerves showed that activity was low during egg-laying but high at other times. The significance of the oviducal contractions observed in *Manduca sexta* is not known but the evidence from other species suggests that it would be reasonable to assume that these too serve to retain eggs until a suitable time for laying.

A further regulatory process of oviduct motility is derived from the male insect. Egg-laying is stimulated by mating in many insects (review: Leopold 1976). Extracts of the male accessory gland are myoactive in the female oviduct of *Locusta migratoria* (Lafon-Cazal *et al.* 1987) and *Manduca sexta* (T. M. Stephens, University of Bath, personal communication) and several peptides which stimulate the oviduct have been isolated from these locust male accessory glands; proctolin (Paeman 1991 in Schoofs *et al.* 1993), locusta-accessory gland myotropin-I (Lom-AG-MT-I, Paeman *et al.* 1991b) and Lom-AG-mytropin-II (Paeman *et al.* 1991c). Corresponding peptide fractions were obtained from spermatophores in females suggesting that male derived oviduct myotropic peptides are transferred to the female during copulation.

The functional significance of the neural and hormonal controls governing oviduct motility are not well understood but clearly the mechanism by which eggs are moved through the reproductive system and how co-ordination of the various components of female reproduction is achieved is complex and warrants further investigation. The experiments here indicate that a peptide with close similarity to CCAP found in the greatest quantity in the nerves DN8 and VN7 is responsible for modulating contractions of the upper common oviduct probably as a circulating neurohormone.

The results from this study of immunohistochemical staining of adult abdominal

nerve cords with anti-CCAP antiserum 2TB are largely in agreement with those of Davies *et al.* (1993) (see Fig. 2.19). These workers found similar numbers of neurons in ganglia 3 – 5 with an additional unpaired median neuron posterior to the first, detected in preparations in which colchicine was used as a pretreatment before dissection. The neurons were identified by comparison to past studies made of the abdominal neurosecretory cells. The following description is an adaption of that given in Davies *et al.* (1993). The paired lateral cells anterior to the root of the dorsal nerve are motor neurons termed MN-1 (motor neuron 1, Levine and Truman 1985) and their axons extend to the contralateral dorsal nerve, terminating on dorsal external muscles (Levine and Truman 1985). One pair from the set of four lateral cells are NS-L_1s. Neurosecretory cells which project, via the dorsal nerve, to the transverse nerve of the next ganglia. The other pair are IN-704s, interneurons with extensive arborisation in the dorsal neuropil and also branches that ascend and descend in the connectives, terminating anteriorly in the tritocerebrum. The pair of midline cells are NS-M_4, neurosecretory cells which project to the transverse nerve of the same ganglion. The unpaired midline cell is a VUM (ventral unpaired median) cell, one of two VUMs (Pfluger *et al.* 1993) which extend axons via the ventral nerve to the transverse nerve of the next ganglion.

Although this study is largely in agreement with Davies *et al.* (1993), surprisingly no note of immunoreactivity in the nerves to the reproductive tract has previously been made (also H. Dircksen, personal communication). A possible but somewhat unlikely explanation may lie in the concentration of these workers in the detailed mapping of intra-ganglionic CCAP immunoreactivity rather than that in peripheral nerves.

4.4.1 Summary

In conclusion, the oviduct of *Manduca sexta* has been shown to be responsive to factors present in the abdominal nerve cord. Synthetic CCAP is myoactive on the oviduct *in vivo* (Chapter III). Extracts of specific nerves exiting the terminal ganglia are myotropic in the oviduct and the same nerves stain with an anti-CCAP immunoreactive antibody. The peptide CCAP is strongly implicated as an oviduct stimulating neuropeptide, probably released from neurohaemal areas on the terminal nerves DN8/transverse nerve 8 and VN7 and PVOs and acting as a circulating neurohormone.

Chapter 5

Endogenous Myoactive Neuropeptides in the Nervous Systems of *Manduca sexta* and *Schistocerca gregaria.*

5.1 Introduction

The use of the HPLC in order to separate and identify peptides has enabled great advances to be made in the understanding of insect neurophysiology in recent years. The number of insect neuropeptides isolated and sequenced is vast in comparison with a few years ago.

In previous chapters of this study, the activity of a number of insect neuropeptides on the heart and the oviduct of *Manduca sexta* has been studied. The number of known endogenous myoactive peptides in this insect is limited in comparison to the number isolated from other insect species. Previous investigations have largely centered on the cockroach *Leucophaea maderae* (Holman *et al.* 1990) and the locust *Locusta migratoria* (review: Schoofs *et al.* 1993). The peptides which have been isolated from *Manduca* (see Chapter II, listed in Table 2.1) form a far smaller set than for example the some 21 neuropeptides isolated and sequenced

from *Locusta migratoria*. Why comparatively few peptides have been isolated from *Manduca* is not entirely clear. To some extent the answer must lie in the emphasis of past work on the cardioactive peptides from the abdominal nerve cord of this insect with less attention being paid to peptides located in the brain and associated structures.

A considerable number of myotropic neuropeptides have been isolated from *Locusta migratoria* (review: Schoofs *et al.* 1993) with the use of the heterologous bioassay, the isolated hindgut of the cockroach, *Leucophaea maderae*. These include structurally unique peptides and peptide families such as locustamyotropins (Schoofs *et al.* 1990 d,e, 1992a), locustapyrokinin (Schoofs *et al.* 1991a), locustakinin (Schoofs *et al.* 1992b), locustamyoinhibiting peptide (Schoofs *et al.* 1991b), schistoFLRFamide (Schoofs *et al.* 1993a), locustamyoinhibin (Schoofs *et al.* 1993c), Lom-AKH-I$^{4-10}$ (Schoofs *et al.* 1993b) and locusta accessory gland myotropins I and II (Lom-AG-MT I and II) (Paeman *et al.* 1991 a,b), and also peptides with structural similarities to vertebrate neuropeptides such as locustasulfakinin (Schoofs *et al.* 1990b), similar to gastrin/cholecystokinin and the locustatachykinins (Schoofs *et al.* 1990 a,c) similar to the tachykinins. The *Leucophaea maderae* hindgut bioassay has also been used in a number of other studies for the same purpose of isolation and structural characterisation of neuropeptides. Examples include; *Acheta domesticus* (Holman *et al.* 1991a), *Neobellieria bullata* (Fonagy *et al.* 1992 c,d) and *Leptinotarsa decemlineata* (Spittaels *et al.* 1991).

Although without doubt, the *Leucophaea maderae* hindgut has been an invaluable aid in the search for insect neuropeptides, the use of a single bioassay must have limitations. It is unlikely that this tissue responds to all myotropic peptides from either conspecific insects or from the other species which have been screened using the bioassay. A number of myoactive fractions have subsequently been applied

to the *Locusta* midgut and oviduct but initial screening has consistently been conducted using the cockroach hindgut bioassay. In comparison, the insect heart has had limited use as a tool for screening for cardioactive neuropeptides and in addition, the activity of most known insect neuropeptides on the heart has not been thoroughly investigated.

The semi-isolated heart of *Manduca* has been employed with great success in studies of the physiological actions of the *Manduca* cardioactive peptides (CAPs and CAFs) and also in the isolation and subsequent sequencing of these peptides. Previous work using the adult and larval heart bioassays has not included a screening of the brain tissues of *Manduca* or investigated the potential of these heart bioassays as screening tools in the search for cardioactive neuropeptides from other species.

The *Manduca* oviduct bioassay is a novel tool for the detection of myotropic neuropeptides. The screening of insect nervous tissues with this bioassay may identify further myoactive peptides and also aid in the understanding of neurohormonal regulation of the motility of the oviduct.

This study describes an investigation into the myoactive factors present in the brain, whole head and nerve cord of both *Manduca sexta* and the desert locust, *Schistocerca gregaria.* An HPLC system was used to separate the peptides and the resultant fractions screened with the adult and larval *Manduca* heart bioassays and the *Manduca* oviduct bioassay (described in Chapter III). A number of synthetic neuropeptides, known to occur in either or both of these insects, were subjected to identical HPLC conditions as the nerve cord extracts. The elution time of the synthetic peptides enabled some predictions as to the identity of active fractions to be made.

5.2 Methods

5.2.1 Insects

The *Manduca sexta* culture has been described elsewhere. The locusts, *Schistocerca gregaria* were maintained at 31 - 35°C with a 12 hour light and 12 hour dark photoperiod. Mature adult insects aged approximately 15 days after the final moult were used in these experiments.

5.2.2 Preparation of samples for HPLC

Adult insects of both sexes were selected and dissected under E and B saline (Euphrussi and Beadle 1936; see Appendix). Heads were severed from the thorax. Antennae and mandibles were removed from the locust heads and antennae, probiscus, eyes and scales were cut from the *Manduca* heads. Brains were left as complete as possible, removing the cuticle and fat body entirely while retaining the retro-cerebral complex (corpora allata and corpora cardiaca) and sub-oesophageal ganglion but not the frontal lobe. Nerve cords were dissected from the abdomen and any remaining fat body was removed. Tissues were frozen immediately after dissection initially in liquid nitrogen and then stored at -40°C until required.

Extraction medium A consisted of methanol (BDH, HPLC grade) 90%, water (double distilled, Millipore filtered) 9% and glacial acetic acid (BDH, HPLC grade) 1% (from Schoofs *et al.* 1990a).

Extraction medium B consisted of 1M acetic acid (BDH, HPLC grade), 20mM

H_2SO_4 (BDH Analar grade), 0.1 mM PMSF (phenyl methyl sulphonyl fluoride) and 1mM EDTA (ethylendiaminetetracetic disodium salt) (adapted from Kataoka *et al.* 1987).

Tissues were homogenised in one of the two extraction media in a chilled ground glass-glass homogeniser resting on ice. Approximately 5ml of the extraction media was used for every ten brains or 20 nerve cords. The homogenate was then centrifuged at 10 000 r.p.m. (IEC Centra-3 centrifuge) for five minutes and the supernatant retained. The precipitate was resuspended in water, recentrifuged and the resultant supernatant pooled with the previous batch.

A Sep-pak (Waters C-18) was previously primed with: 5ml HPLC water, 5ml acetonitrile, 5ml HPLC water then 5ml 10% acetonitrile/0.1% trifluoroacetic acid (TFA, BDH, HyPerSolv HPLC grade) using a Luer fitting syringe. The sample was loaded and the Sep-pak washed with 2ml of the 10% acetonitrile/0.1% TFA. The Sep-pak was eluted with 2ml of 60% acetonitrile/0.1% TFA and the eluate retained for dilution prior to injection onto the HPLC.

5.2.3 HPLC Separation of nervous tissue extracts

The HPLC system used was a Gilson run by a Gilson 715 HPLC controller program. The system had two 306 pumps with 10ml heads. The columns used were: a C-4 Hypersil 10μm (25cm) separating column with a C-4 Hypersil (5cm) guard column, and a C-18 Spherisorb 5μm separating column (25cm) with a C-18 Spherisorb (5cm) guard column (both columns were packed at the University of Bath using a Magnus Scientific P6060 HPLC slurry packer).

The Rheodyne injection port was fitted with a 5ml injection loop, absorbance was measured by a Gilson 115 UV detector set at 210nm. The solvents used to generate the gradient were; Millipore filtered double distilled water containing 0.1% TFA and 100% acetonitrile with 0.1% TFA. The mobile phase was pumped at 1ml min^{-1}. Fractions of 1ml were collected with a Gilson FC 203 fraction collecter.

The HPLC was equilibrated and a blank gradient was run prior to sample injection at the start of each day of tests. Before sample injection the HPLC was equilibrated at 10% acetonitrile/0.1% TFA. Nervous tissue samples were diluted to give a 10% acetonitrile/0.1% TFA concentration and injected in 4ml batches with five minutes delay between each to allow the loop to clear. The synthetic peptide samples were all 1μg in a volume of 100μl of water.

- Separation protocol 1 – using the C-4 column, a gradient of 10% acetonitrile/0.1% TFA to 80% CH_3CN/0.1% TFA was run over 70 minutes, fractions were collected every two minutes giving 2ml fractions except for the *Manduca* brain, for which fractions were collected every one minute.
- Separation protocol 2 – using the C-18 column, a gradient of 10% acetonitrile/0.1% TFA to 50% acetonitrile/0.1% TFA was run over 40 minutes, fractions were collected every minute giving 1ml fractions.
- Separation protocol 3 – using the C-18 column, a gradient of 20% acetonitrile/0.1% TFA to 40% acetonitrile/0.1% TFA was run over 40 minutes fractions were collected every minute giving 1ml fractions.

Fractions were collected as described and aliquots of each transferred to Eppendorf tubes with 20μl of 0.1% bovine serum albumen (BSA, Sigma, heat shock

fractionated grade IV) solution in dH_2O. These were lyophilised overnight prior to storage at - 40°C. The dried fractions were redissolved in either adult or larval *Manduca* saline before use in the bioassays. The remaining volume of each fraction was retained in acetonitrile/water and stored at +4°C until required for further tests.

5.2.4 Bioassay Methods

The bioassay methods used were the adult heart, the larval heart and oviduct bioassays as described fully in Chapters II and III respectively. For the testing of HPLC fractions, in the heart bioassays, 10μl samples were applied and 20μl in the oviduct bioassay. In all cases, 10μl represented 0.25 insect equivalents and 20μl was 0.5 insect equivalents.

5.3 Results

5.3.1 Assessment of Extraction Media.

In initial experiments the efficacy of two extraction media previously used or adapted from those used by other workers in similar studies was examined (extraction medium A – Schoofs *et al.* (1990a), extraction medium B – Kataoka *et al.* (1987)). Ten *Schistocerca gregaria* whole heads were extracted with each media and separated by reverse phase (C-4) HPLC (separation protocol 1). The HPLC chromatograms of absorbance at 210nm during the two separations are shown in Fig. 5.1. Comparison of the traces shows that similar amounts of material eluted from the HPLC however, the observable peaks are not comparable.

The resultant number of fractions which caused modulation of the *Manduca* heart and oviduct bioassays plus the amount of activity in the fractions were used to assess the suitability of the two media. The results of the comparison are shown in Table 5.1. Both extraction media gave four fractions that were active in the larval heart bioassay, but on the adult heart bioassay extraction medium A gave three active fractions whilst B gave five. In the oviduct bioassay, no fraction extracted with medium A was active whereas three fractions were myoactive from the medium B extraction.

The most myostimulatory fraction from the medium B extraction in the adult and larval hearts was that which eluted at 24 – 25 minutes giving a heartrate increase of 101.0% to 150.0% with the 27 – 28 minute fraction being strongly cardiosuppressive on the larval heart, giving a heartrate decrease of 101.0% to 150.0%. The factors extracted by medium A with a retention time of 22 – 23 minutes, were most cardioactive giving a heartrate increase of 51.0% to 100.0% and no cardiosuppressive fractions were noted. In the oviduct the fraction eluting at 22 – 23 minutes was most myoactive with a contraction rate increase of between 401.0% and 600.0%.

As extraction medium B appeared to give both more activity and a larger number of active fractions, it was employed in all subsequent experiments.

5.3.2 First HPLC Separation of Nervous System Extracts.

In further experiments sets of brains, whole heads and abdominal nerve cords from both *Manduca sexta* and *Schistocerca gregaria* were extracted and separated by HPLC using separation protocol 1.

Fraction number (Retention time (mins))	20-21	22-23	24-25	26-27	28-29	30-31	32-33
Extraction medium A							
Larval heart		++	+	−	+		
Adult heart		++	+	+			
Oviduct							
Extraction medium B							
Larval heart		++	+++	− − −	+		
Adult heart		++	+++	++	+	++	
Oviduct		+++	++	++			

Table 5.1: Table showing bioactive fractions obtained from HPLC separation of *Schistocerca gregaria* whole heads using two extraction media.
A – methanol : water : acetic acid 90 : 9 : 1 and B – 1M acetic acid, 20mM sulphuric acid, 0.1mM PMSF, 1mM EDTA. 0.05 (10µl) insect equivalents of each fraction was applied in each test in the heart bioassays and 0.1 (20µl) insect equivalents in the oviduct bioassay. Key to symbols for the adult and larval heart bioassays: + 1 to 50% rate increase, ++ 51 to 100% rate increase, +++ 101 to 150% rate increase, − 1 to 50% rate decrease, − − 51 to 100% rate decrease, − − − 101 to 150% rate decrease and for the oviduct bioassay: + 1 to 200% rate increase, ++ 201 to 400% rate increase, +++ 401 to 600% rate increase.

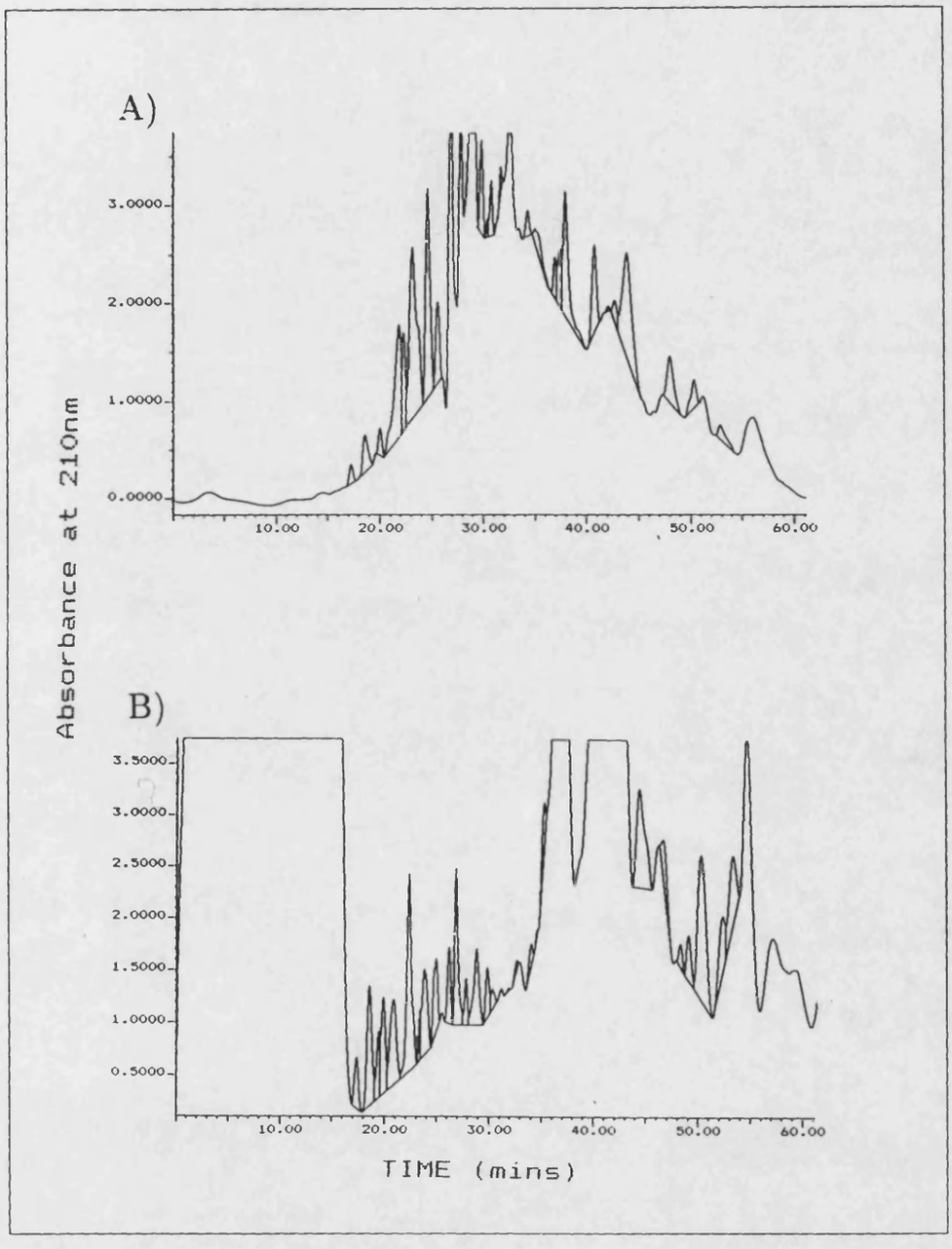

Figure 5.1: Chromatograms of the separation by HPLC of *Schistocerca* whole heads. *A) 10 Schistocerca whole heads using extraction media A and B) 10 Schistocerca whole heads using extraction media B using separation protocol 1, C-4 column. Absorbance measured at 210nm.*

The Bioactivity of Fractions from HPLC Separation 1 of *Manduca sexta* nervous tissue.

The results from the screening of fractions by the bioassays, from the first separation of 20 whole heads, 40 brains and 20 abdominal nerve cords from *Manduca sexta* by HPLC are shown in Tables 5.2, 5.3 and 5.4 and in graph form in Fig. 5.2. The same data is shown in both table and graph form. It should be noted that brain fractions were collected every minute whereas whole head and nerve cord fractions were collected every two minutes.

From the separation of the adult *Manduca* nerve cord extract, seven of the 2ml fractions were cardioactive in the adult heart bioassay while seven fractions caused cardioacceleration in the larval heart bioassay and one fraction was cardioinhibitory in the larval bioassay. In the oviduct bioassay, two fractions were myoactive. Two main sets of fractions containing peptides cardioactive in the adult heart were obtained and one main set of fractions that were active in the larval heart with a second less active fraction coinciding with the second adult cardioactive fraction. A single peak of activity was seen in the oviduct bioassay, coinciding with the first peak of cardioactivity noted in the adult and larval heart assays.

From the adult *Manduca* head extract, seven fractions were obtained that were cardioactive on the adult heart, nine fractions were active on the larval heart and three on the oviduct. No cardiosuppressive fractions were noted. The two peaks of activity in all three bioassays coincided largely, but not exactly, with the two peaks of cardioactivity seen in responses of the heart bioassays to the nerve cord extracts.

Fraction number	*Manduca sexta* brain	*Manduca sexta* whole head	*Manduca sexta* nerve cord
20	+	++	-
21	+++		
22	++++	++	
23	- -		
24	- -	++	++
25	+++		
26	+	++	++
27	++		
28	++	++++	++
29	++		
30	+	+	+
31			
32	+	+	++
33	++		
34	++	++	+
35	+		
36	++	+	
37	++		

Table 5.2: Table showing responses of the adult heart bioassay to HPLC fractions of adult *Manduca* nervous tissues.
0.05 (10μl) insect equivalents of each fraction was applied in each test. For key to symbols see caption of Table 5.1.

In the adult *Manduca* brain, seven fractions were found to be active in the adult heart, 17 in the larval heart and three in the oviduct. Of the active fractions two were cardiosuppressive in the larval heart but were not active in the adult heart or oviduct. No cardiosuppressive fractions were found in the whole head or nerve cord extracts. A set of fractions which eluted earlier than others and with strong myoactivity in all three bioassays was noted. This activity was not detected in nerve cord extracts. In whole head extracts, the corresponding fractions were active on the larval heart but had no activity in the oviduct and very little in the adult heart.

Fraction number	*Manduca sexta* brain	*Manduca sexta* whole head	*Manduca sexta* nerve cord
20		+	
21	+		
22	++		+
23			
24		+	+
25			
26	++	+	+++
27	+++		
28	++	++	+++
29	+		
30	+		+
31			
32		+++	+++
33			
34		++	++
35			
36		+	
37			

Table 5.3: Table showing responses of the larval heart bioassay to HPLC fractions of adult *Manduca* nervous tissues.
0.05 (10μl) insect equivalents of each fraction was applied in each test. For key to symbols see caption of Table 5.1.

Fraction number	*Manduca sexta* brain	*Manduca sexta* whole head	*Manduca sexta* nerve cord
20			
21			
22	+++		
23			
24			
25			
26	+	+	++
27	++		
28		++	+++
29			
30			
31			
32		+	
33			
34			
35			
36			
37			

Table 5.4: Table showing responses of the oviduct bioassay to HPLC fractions of adult *Manduca* nervous tissues.
0.1 insect equivalents were applied of each fraction (20µl). For key to symbols see caption of Table 5.1.

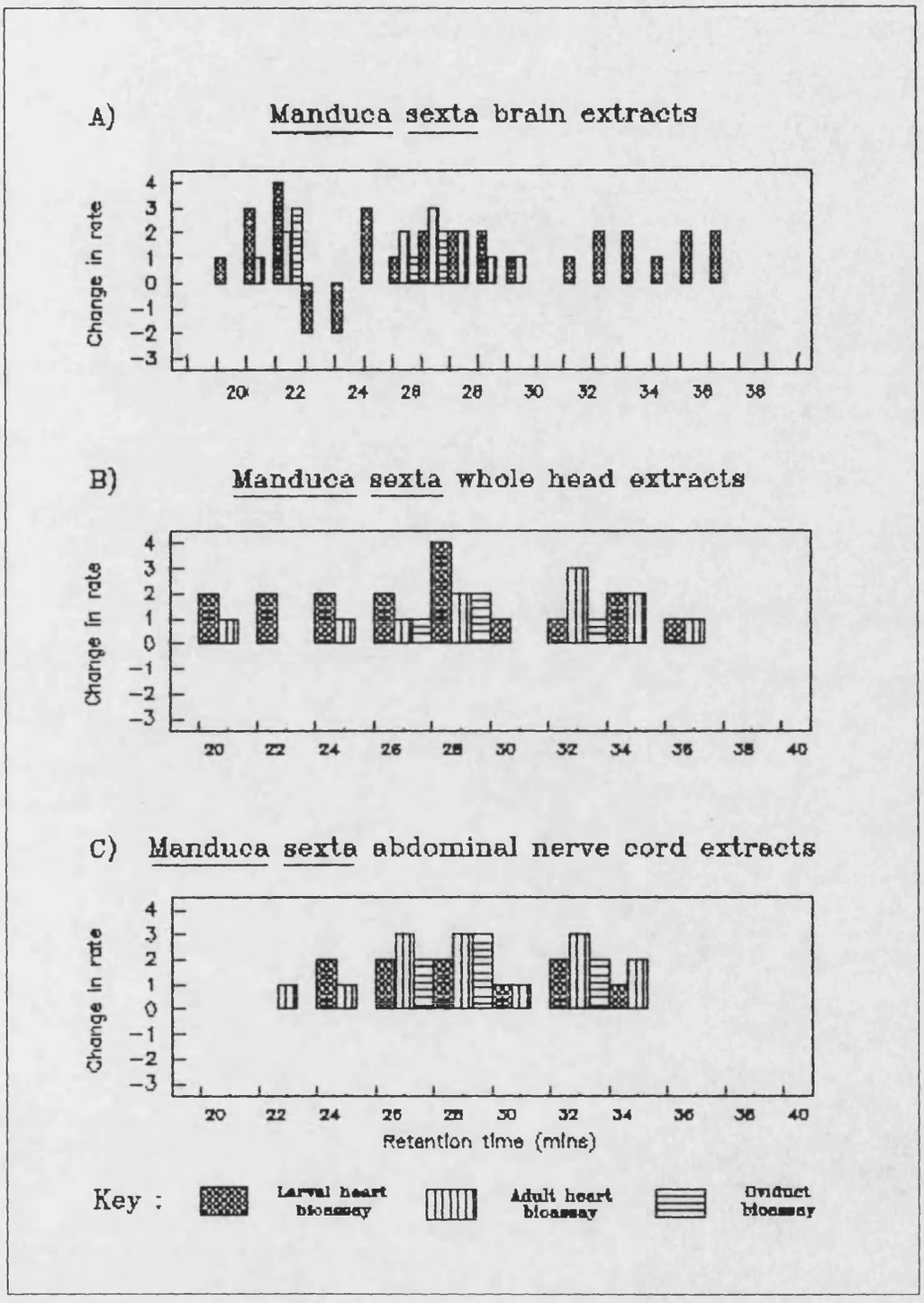

Figure 5.2: Graph showing the response of the larval and adult hearts to HPLC fractions from extracts of *Manduca sexta* brain, whole head and abdominal nerve cord.

0.05 (10µl) insect equivalents of each fraction was applied in each test. Rate change represented by: 0 to 1 – 1 to 50% rate change, 1 to 2 – 51 to 100% rate change, 2 to 3 – 101 to 150% rate change, 3 to 4 – 151% to 200% rate change.

The Bioactivity of Fractions from HPLC Separation 1 of *Schistocerca gregaria* nervous tissue.

The chromatograms obtained from the separation of *Schistocerca* nervous tissues by HPLC are shown in Figs. 5.1 and 5.3. The results from the screening by the *Manduca* bioassays of the fractions from the HPLC separation of ten whole heads, ten brains and 20 abdominal nerve cords from *Schistocerca gregaria* are shown in Tables 5.5, 5.6 and 5.7 and in Fig. 5.4. The same data is shown in both table and graph form. It should be noted that whereas fractions from the brain and nerve cord separations are equivalent, the whole head fractions are out of synchronisation by 1 minute. Therefore, the factors present in the fraction collected over two minutes are not directly comparable between the brain/nerve cord separation and that of the whole head.

The separation of the locust nerve cord extract produced five fractions cardioactive in both the larval and adult heart assays and three myoactive in the oviduct bioassay. The whole head extracts contained five fractions active in the larval heart, four active in the adult and three in the oviduct. The brain extracts contained four cardioacceleratory fractions in the adult heart, six active in the larval and two myostimulatory in the oviduct. In both the brain and the whole head separations, one fraction was cardiosuppressive in the larval heart. This fraction did not have the same retention time as the cardiosuppressive fraction from *Manduca* brains. No cardiosuppressive fractions were noted in the nerve cord extracts.

Elution of Synthetic Peptides with HPLC Separation Protocol 1.

The synthetic peptides *Manduca sexta* allatotropin (Mas-AT), crustacean cardioactive peptide (CCAP), corazonin, locustatachykinin II (Lom-TK II) and schistoFLRFamide were subjected to the same HPLC separation (separation protocol 1) as the insect nervous tissues. In separate HPLC runs for each peptide, the peak elution time was recorded and the fraction number identified with use of the bioassays. The chromatogram of the five peptides is shown in Fig. 5.5. This data gave a good indication of where these peptides would be expected to occur if present in the nervous tissue extracts. Table 5.8 gives the peak maxima at the detector and the expected elution times at the fraction collecter are shown. The fraction number in which each peptide was detected by bioassay, either adult heart bioassay for Mas-AT or larval heart bioassay for CCAP, corazonin, Lom-TK II and schistoFLRFamide is also shown. The delay from detector to fraction collecter was found to be close to 2.5 minutes.

5.3.3 Second and Third HPLC Separation of Nervous System Extracts.

Selected active fractions from the first C-4 (separation protocol 1) HPLC separation of both *Manduca* and *Schistocerca* brains were subjected to further HPLC analysis using a C-18 column.

Fraction number	*Schistocerca gregaria* brain	*Schistocerca gregaria* whole head	*Schistocerca gregaria* nerve cord
20			
21	+		
22		++	
23	++		++
24		+++	
25	+++		+++
26		- - -	
27	- - -		+
28		+	
29	++		++
30			
31	++		++
32			
33			
34			
35			
36			
37			

Table 5.5: Table showing responses of the larval heart bioassay to HPLC fractions of adult *Schistocerca gregaria* nervous tissues.
0.05 (10µl) insect equivalents of each fraction was applied in each test. For key to symbols see caption of Table 5.1.

Fraction number	*Schistocerca gregaria* brain	*Schistocerca gregaria* whole head	*Schistocerca gregaria* nerve cord
20			
21			
22		++	
23			++
24		+++	
25	++		+++
26		++	
27	+++		+++
28		+	
29	++		+++
30		++	
31	++		+++
32			
33			
34			
35			
36			
37			

Table 5.6: Table showing responses of the adult heart bioassay to HPLC fractions of adult *Schistocerca gregaria* nervous tissues.
0.05 (10µl) insect equivalents of each fraction was applied in each test. For key to symbols see caption of Table 5.1.

Fraction number	*Schistocerca gregaria* brain	*Schistocerca gregaria* whole head	*Schistocerca gregaria* nerve cord
20			
21			
22		+++	
23			++
24		++	
25	+++		+++
26		++	
27	++		+++
28			
29			
30			
31			
32			
33			
34			
35			
36			
37			

Table 5.7: Table showing responses of the *Manduca* oviduct bioassay to HPLC fractions of adult *Schistocerca gregaria* nervous tissues.
0.1 insect equivalents were applied of each fraction (20μl). For key to symbols see caption of Table 5.1.

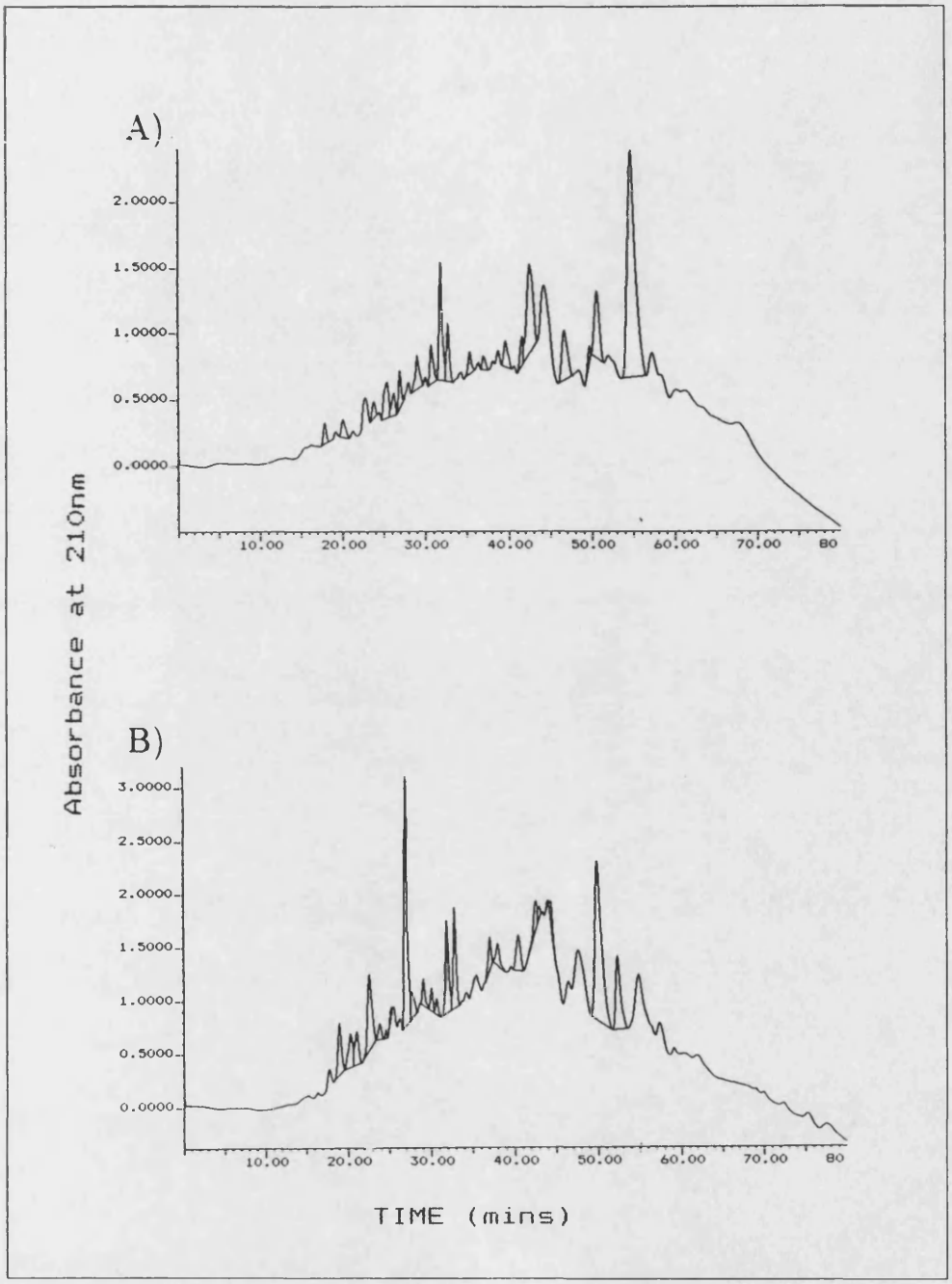

Figure 5.3: Chromatograms of the separation of *Schistocerca* brains and nerve cord. *A) 10 Schistocerca brains and B) 20 Schistocerca abdominal nerve cords by HPLC using separation protocol 1, C-4 column. Absorbance measured at 210nm.*

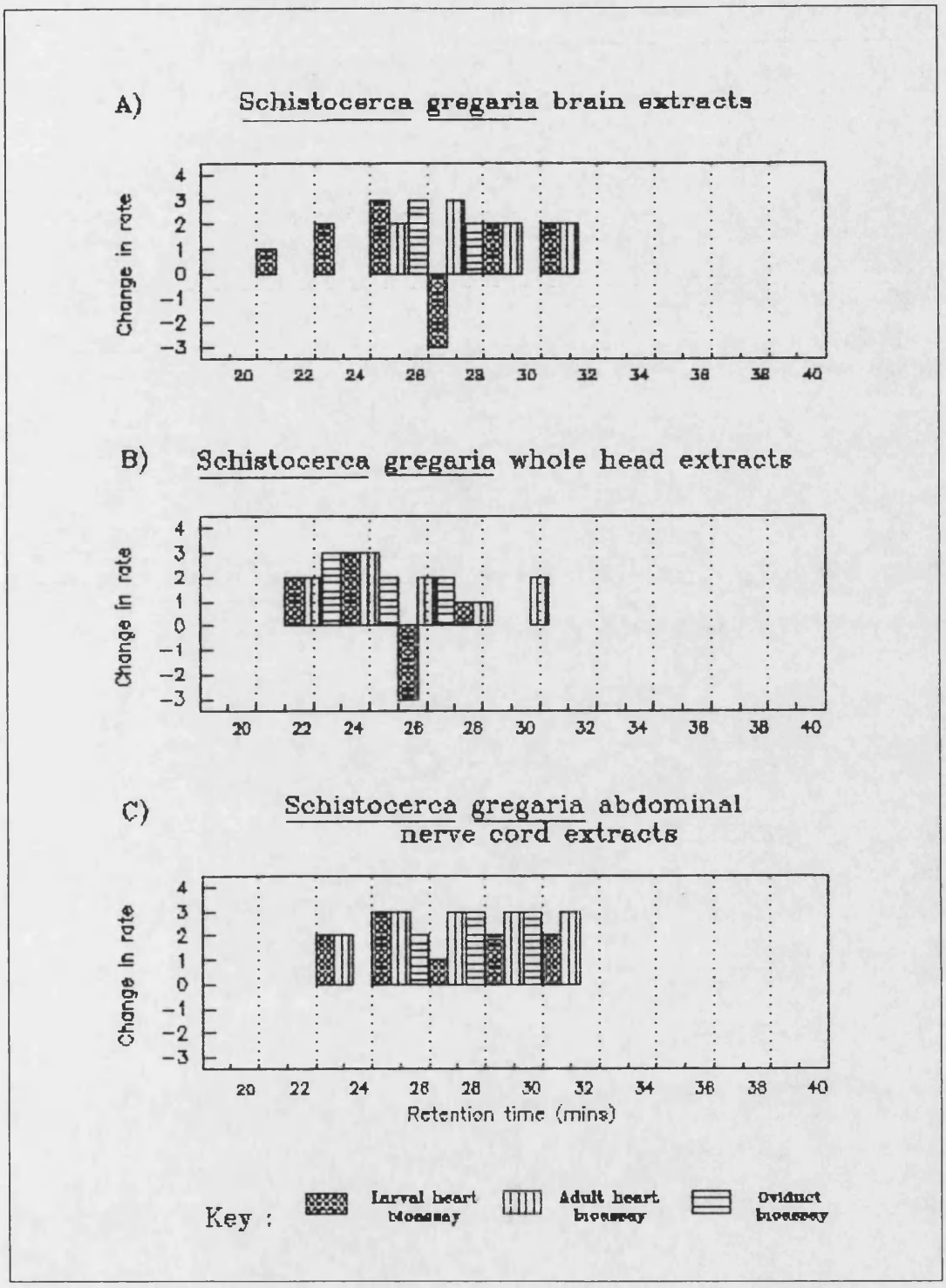

Figure 5.4: Graph of the response of the larval and adult hearts to HPLC fractions from extracts of *Schistocerca gregaria* brain, whole head and abdominal nerve cord. *0.05 (10µl) insect equivalents of each fraction was applied in each test. Rate change represented by: 0 to 1 – 1 to 50% rate change, 1 to 2 – 51 to 100% rate change, 2 to 3 – 101 to 150% rate change, 3 to 4 – 151% to 200% rate change.*

Peptide	Peak time (mins)	Elution time at fraction collecter	Fraction number (Retention time)
Mas-AT	27.51	30.01	31 – 32
CCAP	25.37	27.87	28 – 29
Corazonin	21.97	24.47	25 – 26
Lom-TK II	24.37	26.87	27 – 28
SchistoFLRFamide	30.46	32.96	33 – 34

Table 5.8: Table showing the time of the peak maxima and the expected elution time at the fraction collecter for each of five synthetic peptides using extraction protocol 1.
Also shown is the fraction number (retention time) in which peptide was detected either by adult heart bioassay (Mas-AT) or larval heart bioassay (CCAP, corazonin, Lom-TK II and schistoFLRFamide). Conditions as separation 1 protocol (see text for details).

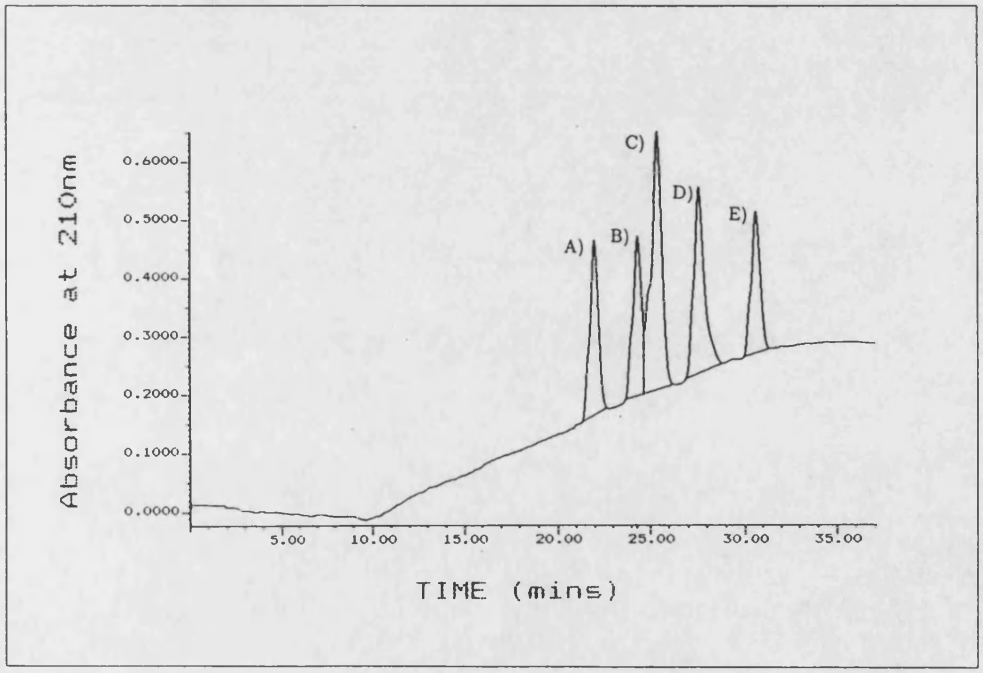

Figure 5.5: HPLC chromatogram of 5 synthetic peptides separated using separation protocol 1.
A) corazonin, B) Lom-TK II, C) CCAP, D) Mas-AT and E) schistoFLRFamide. Approximately 1ug of each peptide was injected.

Sep. 1	Sep. 2	Larval heart	Adult heart	Oviduct
23 – 24	27	–		
	28	– – –		
	29	– –		

Table 5.9: Table showing responses of the *Manduca* larval and adult heart and oviduct to fractions from the 2nd separation of *Manduca sexta* brain extracts. *Separation protocol 2. For key to symbols see caption of Table 5.1.*

Manduca sexta **Nervous Tissues.**

From the *Manduca* brains the cardiosuppressive fractions in the larval heart were of particular interest as there have been no previous reports of cardioinhibitory peptides from this insect. The fractions 23 and 24 were pooled and separated with the separation protocol 2 and the resultant chromatogram is shown in Fig. 5.6. The results are shown in Table 5.9. In the larval heart, three of the resultant fractions were cardiosuppressive.

Schistocerca gregaria **Nervous Tissue.**

In the locust, fractions 23 – 24, 25 – 26 and 27 – 28 of the brain extract were further separated with the separation protocol 2. Chromatograms of the second separation are shown in Fig. 5.7. The results are shown in Table 5.10. Fraction 23 – 24 gave three fractions with some cardioactivity in the larval heart. Fraction 25 – 26 gave 2 fractions with myoactivity in larval, adult and oviduct bioassays and one with activity in only the larval and adult. Fraction 27 – 28

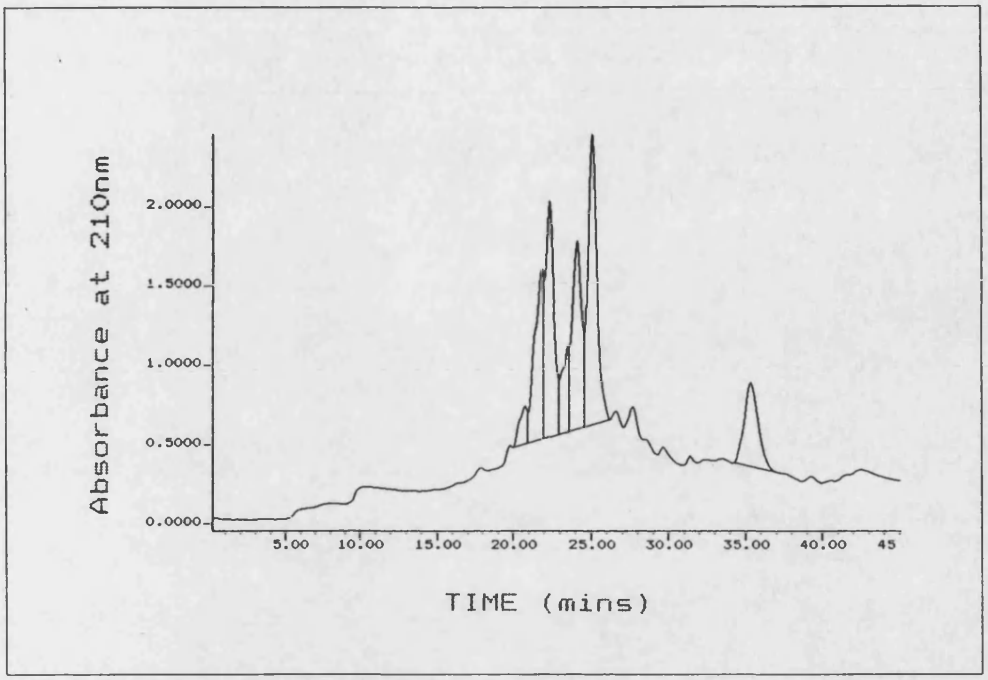

Figure 5.6: Chromatogram of fractions 23 -24 from the first separation of *Manduca* brains separated for the second time.
Using separation protocol 2.

only in the oviduct, 1 myoactive in the larval heart and oviduct, 1 active in all three bioassays, 1 cardiosuppressive in the larval heart but stimulatory in the adult and oviduct bioassays and 2 further cardiosuppressive fractions on the larval heart. After pooling the 3 larval heart cardiosuppressive fractions a third separation using separation protocol 3 was made. The larval cardiosuppressive factors eluted in 2 successive fractions. Also detected were 3 stimulatory fractions in the larval heart one of which was also stimulatory in the adult heart and fractions stimulatory in the adult heart of which 1 was myoactive in the oviduct.

The responses of the larval heart to cardiosuppressive fractions from the second separation of both locust and *Manduca* extracts are shown in Fig. 5.10.

Elution of Synthetic Peptides with Separation Protocols 2 and 3.

The synthetic peptides CCAP, Lom-TK II and Mas-AT were subjected to the same HPLC separation as used in the separation protocols 2 and 3. The resultant chromatogram from separation protocol 2 is shown in Fig. 5.9. The fraction numbers, equivalent to those of the second and third separations of nervous tissue, at which the synthetic peptides eluted, are shown in Table 5.11.

Sep. 1	Sep. 2	Larval heart	Adult heart	Oviduct	Sep. 3	Larval heart	Adult heart	Oviduct
23 – 24	25	+						
	26	+						
	27	+						
25 – 26	28	++	+	+++				
	29	+++	++	+++				
	30							
	31	++	+					
27 – 28	26			+				
	27							
	28	+		++				
	29	++	++	+++				
	30	–	+++	+	22	+		
	31	– – –			23	+		
	32	– –			24			
					25			
					26	++	+	
					27		++	
					28	–	++	
					29	– – –		

Table 5.10: Table showing responses of the *Manduca* larval and adult heart and oviduct to fractions from the 2nd and 3rd separations of *Schistocerca gregaria* brain extracts.
For key to symbols see caption of Table 5.1.

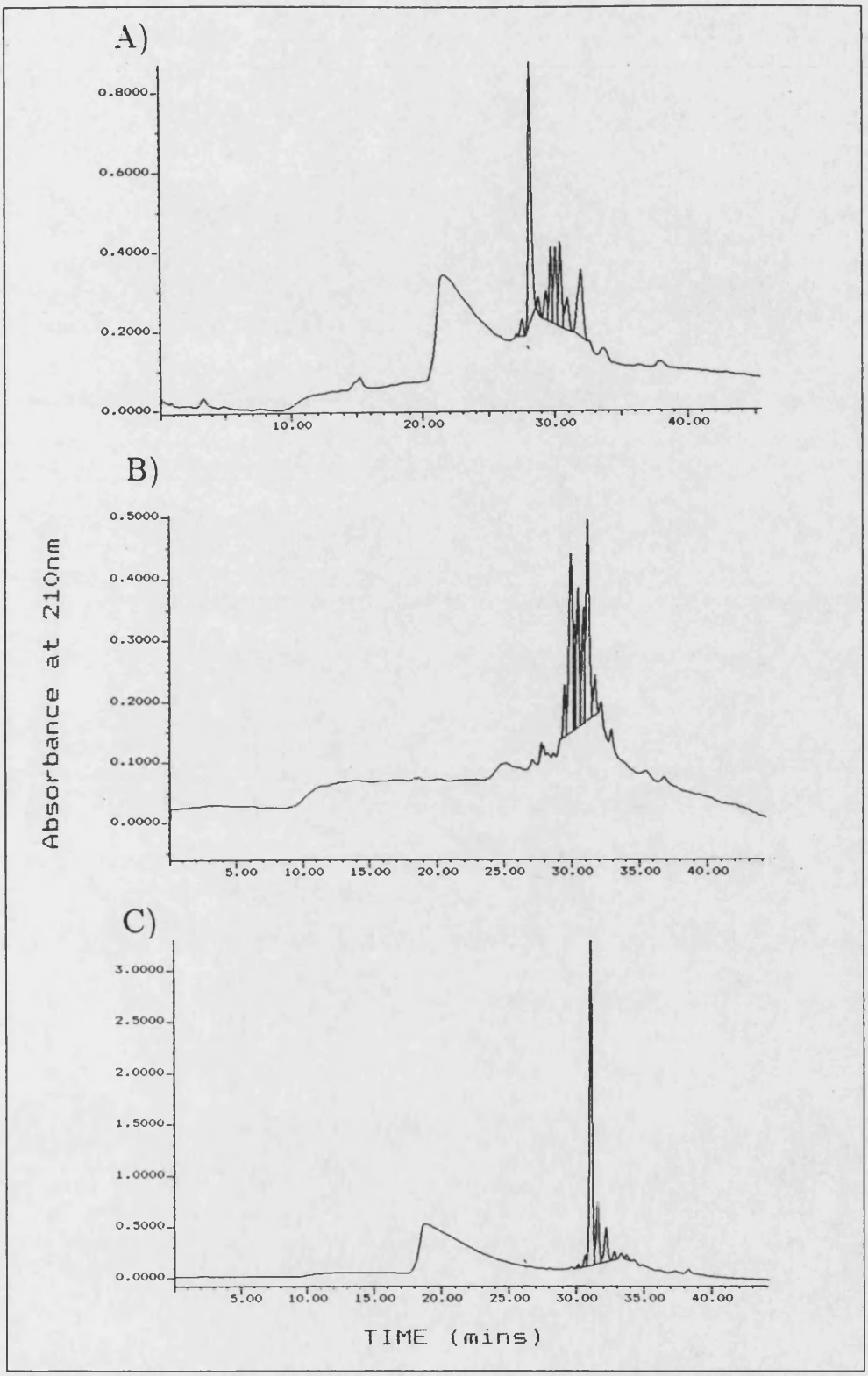

Figure 5.7: Chromatograms of the second separation of selected fractions from *Schistocerca* brain extracts.
A) fractions 23 – 24, B) fractions 25 – 26 and C) fractions 27 – 28 from locust brains. Separation protocol 2, C-18 column.

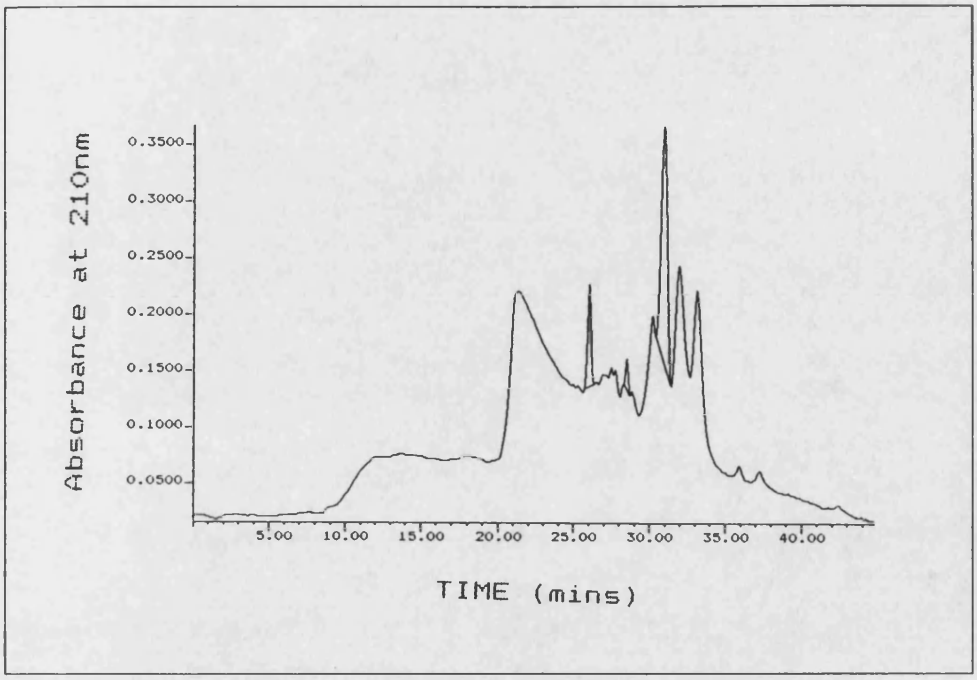

Figure 5.8: Chromatogram showing the third separation of fractions from *Schistocerca* brain extracts.
Fractions 30, 31 and 32 of the second separation, using separation protocol 3.

Peptide	Separation Protocol 1	Separation Protocol 2	Separation Protocol 3
Mas-AT	31 – 32	32 – 33	
CCAP	28 – 29	28 – 29	26
Lom-TK II	27 – 28	27 – 28	22

Table 5.11: Table showing the fraction number in which three synthetic peptides likely or known to occur in either *Manduca sexta* or *Schistocerca gregaria* eluted from the HPLC when separation protocols 2 and 3 were employed.

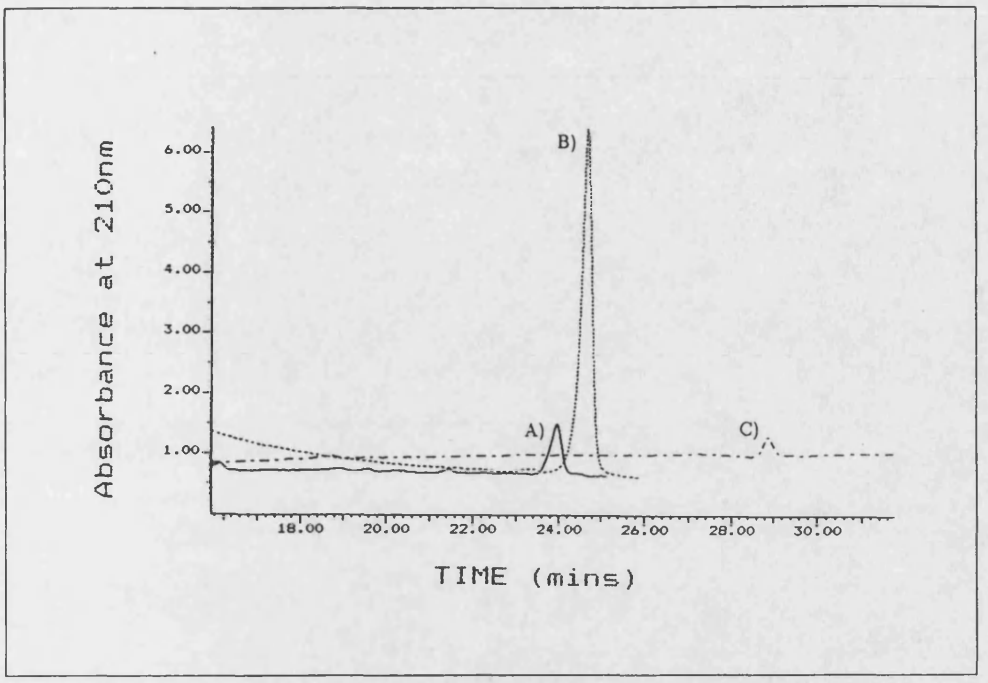

Figure 5.9: Chromatogram of synthetic peptides separated by separation protocol 2. *A) Lom-TK II (0.5μg), B) CCAP(1μg) and C) Mas-AT (0.4μg).*

5.4 Discussion

Previous studies of the myoactive peptides from HPLC separation of *Manduca sexta* adult abdominal nerve cords has revealed one fraction active in the larval heart, the CAP2 peptides (Tublitz *et al.* 1992), and two in the adult, the CAP1 and 2 peptides (Tublitz and Truman 1985, Tublitz and Evans 1986). The CAP2 peptides are now known to consist of a set of three peptides of which CCAP is one, and the CAP1s are a set of two peptides. The results from this study are only in partial agreement with previous work. Responses of both the adult and larval hearts show two main peaks of cardioactivity with the adult heart being relatively more responsive to both. The first peak is likely to be the CAP2 peptides and the second the CAP1 peptides. The oviduct was responsive to one main peak which coincided with the first peak of cardioactivity detected by the

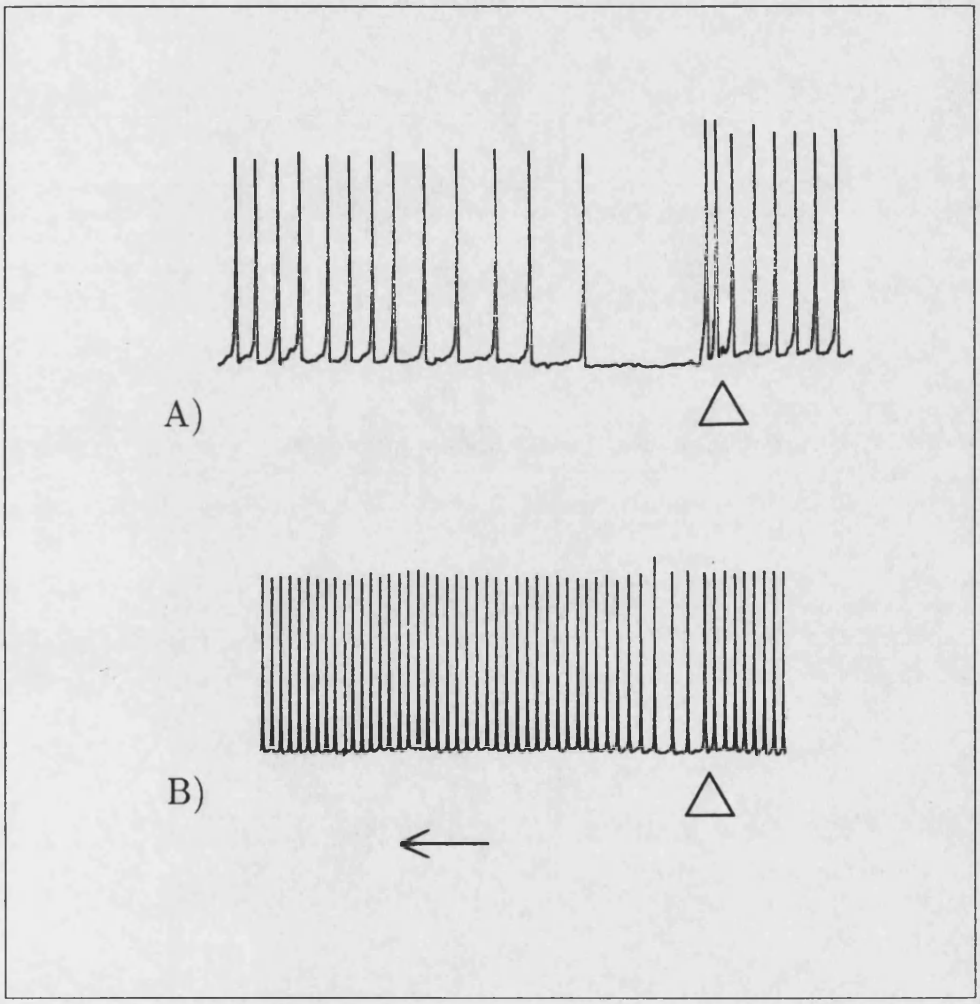

Figure 5.10: Traces from the larval heart bioassay showing typical responses to inhibitory fractions from the second separation of nervous tissues.
A) Schistocerca gregaria extracts – fraction 31, B) Manduca sexta extracts – fraction 28. 0.25 insect equivalents (10μl) was applied. Results from different heart preparations are shown.

heart bioassays. Activity in all the bioassays was spread over several fractions, indicating the presence of more than one peptide, this is in accordance with Cheung *et al.* (1992) who found two CAP1 peptides and three CAP2 peptides. The greater response of the adult heart bioassay to the putative CAP2 peptides in this study may indicate the presence of a comparatively larger amount of the CAP2b and c peptides in the adult nerve cords. These peptides have been shown to be more cardioactive in the adult heart (see Chapter II) than the larval. These results add support to the suggestion put forward in Chapter II, that the members of the CAP2 peptides play different roles in the adult and larval stages.

Why the larval heart responded to peptides eluting at the same time as the presumed CAP1 peptides is not clear. This has not been observed in previous studies, possibly the Kataoka *et al.* (1987) extraction medium used here may extract additional peptides not previously seen since other studies have employed more simple media such as methanol alone (Platt and Reynolds 1985) or acidified methanol (Tublitz *et al.* 1992). The presence of a fraction with slight cardiosuppressive activity in the larval heart has also not been noted previously (Tublitz *et al.* 1992). This factor may have been missed if not separated from cardiostimulatory factors.

The separation of the *Manduca* brains and whole heads has been particularly rewarding in this investigation. In addition to a cardioinhibitory factor in the larval heart, a large number of other fractions were found to be myoactive. The restrictions of time did not allow a thorough investigation to be made, however the importance of further work directed towards the extraction and isolation of peptides from adult *Manduca* heads is demonstrated by these results.

A number of known insect neuropeptides were subjected to HPLC analysis under

identical conditions to the nervous system extracts. The elution times of these synthetic peptides provide a basis from which to predict the identity of some of the myoactive peptides detected in the nervous tissues.

Synthetic Mas-AT eluted in fraction numbers 31 – 32 using separation protocol 1. This peptide known not to be cardioactive in the larval heart (see Chapter II and Veenstra *et al.* 1994) and the results from this study show that although some myoactivity in the larval heart is present in these fractions, particularly in the nerve cord, the response of the adult heart is comparatively greater. A factor with strong cardioacceleratory properties in the adult heart eluted in fractions 32 – 33 from the whole head extracts and nerve cord but was not detected in the brain. The same fractions in the head extracts also contained a factor slightly myostimulatory in the oviduct. These results indicate that a peptide present in whole head extracts, detected by the oviduct and adult heart bioassays, is probably endogenous Mas-AT and that this peptide elutes from the same fractions as identified as containing the CAPIs. That the larval heart also responded to peptides in these fractions suggests that more than one peptide is present, however the much greater response of the adult heart indicates that Mas-AT is likely to be a major component. The putative Mas-AT was not detected in the brain extracts possibly due to loss of some retrocerebral complexes during dissection. In addition the possible Mas-AT activity in the oviduct was not observed from the abdominal nerve cords.

Results from this study support the suggestion made by Veenstra *et al.* (1994) that Mas-AT is a CAP1 in as far as a peptide myoactive in both the adult heart and oviduct elutes in the same fractions as Mas-AT from the brain extracts. However, no conclusive evidence of the presence of this peptide in nerve cords was seen. The peptide concentration in the nerve cord extracts may have been

too low to detect, Mas-AT may be a minor component of the CAPIs in the nerve cord. A repeat of this experiment with an increased number of nerve cords would help in the confirmation of the identity of this peptide as one of the CAPIs.

Synthetic CCAP eluted in fractions 28 – 29. In the larval heart bioassay, a highly cardioactive peak was detected in the whole heads extract in the fraction 28 – 29. The same fractions from the brain and nerve cord extracts were also active but to a lesser extent. The adult heart was responsive to factors present in fractions 28 – 29 with the greatest activity from the nerve cord extracts. In the oviduct, fractions 28 – 29 contained myoactive factors from the whole head and nerve cord but not the brain. These results suggest that CCAP was present in these fractions with most in the nerve cords, then the head extracts and the least in the brain as determined by the adult heart and oviduct bioassays. Both the brain and the nerve cord have been shown to contain CCAP (Lehmen *et al.* 1993) although less occurs in the brain. Davies *et al.* (1993) only detected three pairs of CCAP immunoreactive neurones in the brain-suboesophageal complex in adult *Manduca* whereas each abdominal nerve cord ganglion contained ten neurones with more present in the fused terminal ganglia. A possible reason for the difference in activity between the brain and whole head extracts may lie in the loss of some suboesophageal ganglia during the further dissection of the brain. This structure contains two CCAP immunoreactive neurones (Davies *et al.* 1993).

Synthetic corazonin eluted from this system in fractions 25 – 26. Corazonin has been shown to be present in *Manduca* by immunological methods but has not been isolated from this insect however, [His^7] corazonin has been isolated from *Schistocerca gregaria* (Veenstra 1991). This peptide has been shown to be cardioacceleratory in both the adult and larval hearts but is not active in the oviduct of *Manduca* (see Chapter II). Factors cardioactive in the adult and

larval heart bioassays were obtained from both the *Manduca* and the *Schistocerca* nervous tissues in these fractions, however a discrete peak was not seen and peptides present in fraction 26 were myoactive in the oviduct indicating either that the cardioactive peptide in this fraction is not corazonin or that more than one peptide is present. Identification of the peptides eluting in these fractions would require further investigation.

Synthetic locustatachykinin II (Lom-TK II) eluted from this system in fractions 27 – 28. The family of five tachykinin- like peptides were originally isolated from the locust *Locusta migratoria* (Schoofs *et al.* 1990a, c). Further tachykinins from other insect species, sialokinin I and II from the salivary glands of the mosquito *Aedes aegypti* (Champagne *et al.* 1994) and the callitachykinins I and II from *Calliphora vomitoria* (Lundquist *et al.* 1994) have been isolated and it would seem reasonable to assume that this family of peptides is widespread in insects.

Lom-TK II is cardioactive in the larval heart of *Manduca* but is not active in the adult or oviduct (see Chapters II and III). The separation of fractions in this study was not sufficient to be able to detect any peptides with a similar distribution of activity in these fractions. Further isolation of this fraction would be required to determine the characteristics of the peptides.

Synthetic SchistoFLRFamide eluted from this system in fractions 33 – 34. This peptide was first isolated from *Schistocerca gregaria* (Robb *et al.* 1989) and has subsequently also been isolated from *Locusta migratoria* together with four other structurally related peptides (Lange *et al.* 1994). Members of this family are also present in *Leucophaea maderae* (Holman *et al.* 1986d), *Neobellieria bullata* (Fonagy *et al.* 1992b) and *Drosophila melanogaster* (Nichols 1992). Mas- FLR-Famide has been isolated from *Manduca* (Kingan *et al.* 1990) and two additional

extended FLRFamides (Witten *et al.* 1993).

In Chapter II the shallow dose-response curves show that neither the adult or larval *Manduca* hearts are very responsive to either schistoFLRFamide or Mas-FLRFamide with a threshold doses of 10^{-8} M to 10^{-9} M and 10^{-9} M to 10^{-10} M respectively. The oviduct does not respond to this peptide (see Chapter III). In the *Schistocerca* nervous tissues, no myoactive factors were present in the fractions which correspond to these peptides, a finding is likely to reflect the rather high doses required to elicit a response in the *Manduca* heart. Interestingly, factors cardioactive in the larval heart were present in these and later fractions in the *Manduca* whole head, nerve cord and particularly brain, extracts and in the whole head and nerve cord detected by the adult heart. As the threshold dose of Mas-FLRFamide is similar to that of schistoFLRFamide, it would seem unlikely that Mas-FLRFamide would be detected in *Manduca* nervous tissues and schistoFLRFamide not detected in those of the locust. However, greater numbers of *Manduca* whole heads and brains were used in the initial extract in comparison to the locust extracts, although the insect equivalents applied to the bioassays were the same. Possibly larger amounts of Mas-FLRFamide were present in the *Manduca* extracts than schistoFLRFamide in the locust extracts. Only further investigation of these late-eluting peptides could reveal their identity.

In this study, a major factor cardiosuppressive in the larval heart, from *Manduca* brain extracts was taken through two HPLC separation steps. A different factor, also cardiosuppressive in the larval heart was detected in the *Schistocerca* head and brain extracts taken through three HPLC separation steps. This factor did not elute in the same fractions as that from *Manduca* therefore the peptides concerned are unlikely to be the same in the two insects, but share the characteristic of being cardioinhibitory in the larval *Manduca* heart.

These cardiosuppressive factors are of particular interest because no cardioinhibitory peptides have previously been reported from *Manduca* and no peptide isolated from the locust, which has been applied to the *Manduca* heart bioassays has proved to be cardioinhibitory (see Chapter II). Of the limited range of known insect neuropeptides tested in Chapter II, only the callatostatins from the blowfly, *Calliphora vomitoria* (Duve *et al.* 1993), LPLRFamide (Dockray *et al.* 1983) and leucomyosuppressin (Lem-MS) (Holman *et al.* 1986d) had an inhibitory effect on the larval heart rate at physiological concentrations. These peptides do not elicit the same responses in the larval heart bioassay (see Chapter II, Fig. 2.18. The effect of Met-callatostatin is to cause a long term reduction in beat rate lasting several minutes. LPLRFamide causes a short term cessation of the heartbeat followed by a rapid return to the basal rate within a minute. Application of Lem-MS results in an initial brief increase in rate then inhibition followed by a period in which the heartrate is depressed lasting for approximately a minute. Typical responses of the larval heart to inhibitory fractions from both locust and *Manduca* brains show some similarity with the response to Lem-MS (see Fig. 5.10), that is, initial cardioacceleration, followed by inhibition and a term of slowed heartrate before recovery. The pattern of response did not alter after further HPLC analysis of the fractions suggesting that the response profile was due to cardioinhibitory peptides alone rather than in conjunction with cardioacceleratory peptides.

A possible candidate peptide responsible for the observed cardioinhibition could be the allatostatin from *Manduca*, *Manduca sexta* allatostatin (Mas-AS) (Kramer *et al.* 1991) however, this peptide has no sequence resemblance to the callatostatins (see Fig. 2.8). The action of Mas- AS on the larval heart of *Manduca* has not been reported however some work has suggested that this peptide is not cardioactive in *Manduca* (D. Schooley, personal communication to S. E. Reynolds).

Unfortunately this peptide was not available to test in this study.

The myoactive peptides from the locust, *Locusta migratoria* have been examined in some depth (review: Schoofs *et al.* 1993c) and a number of peptides with inhibitory activity on the *Leucophaea maderae* hindgut and/or the *Locusta migratoria* oviduct have been isolated from this species; locustamyoinhibiting peptide (Lom-MIP) (Schoofs *et al.* 1991b), schistoFLRFamide (Robb *et al.* 1989) (also termed locustamyosuppressin (Schoofs *et al.* 1993a)), ADVGHVFLRFamide (Peeff *et al.* 1994) and locustamyoinhibin (Schoofs *et al.* 1993c). In the cockroach, *Leucophaea maderae* the myoinhibiting peptide Lem-MS has been identified (Holman *et al.* 1986d) which has some sequence similarity to the FLRFamides from *Locusta migratoria.* The activity of these peptides on the heart of these insects has not been reported. Table 5.12 details the sequences of the known myoinhibitory peptides from other insects also included is that of Mas-AS. Interestingly locustamyoinhibin and Mas-AS have four amino acid residues in common which may indicate a structural family link between these two peptides.

Neither schistoFLRFamide or the similar Mas-FLRFamide are cardiosuppressive in the larval *Manduca* heart whereas Lem-MS, also with a FLRFamide carboxy-terminal is cardioinhibitory (see Chapter II). The peptide FPLRFamide is also cardiosuppressive in the larval heart bioassay, therefore, the endogenous cardioinhibitory peptides in *Manduca* could be structurally similar to Lem-MS or FPLRFamide. The larval heart shows a biphasic response to Lem-MS with an initial cardioacceleration followed by inhibition whereas FPLRFamide gives a monophasic response of inhibition alone (Fig. 2.18). The pattern of the larval heartbeat in response to the inhibitory fractions from the nervous tissue extracts were similar to that caused by Lem-MS, suggesting that a peptide structurally more similar to Lem-MS than LPLRFamide, may be present in *Manduca.*

Schisto-FLRFamide	Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe- NH_2
	Ala-Asp-Val-Gly-His-Val-Phe-Leu-Arg-Phe-NH_2
Lem-MS	pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2
Locusta-myoinhibiting peptide	Ala-Trp-Gln-Asp-Leu-Asn-Ala-Gly-Trp-NH_2
Locusta-myoinhibin	pGlu-???-Tyr-???-Lys-Gln-Ser-Ala-Phe-Asn-Ala-Val- Ser-NH_2
Mas-AS	pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile- Ser-Cys-Phe-OH

Table 5.12: Table showing the sequences of insect neuropeptides known to have myoinhibitory activity (in the *Leucophaea* hindgut and/or the *Locusta* oviduct). *Included also is the sequence of Mas-allatostatin (Mas-AS), an identified peptide from Manduca which may be inhibitory on the larval heart and be responsible for the cardioinhibition detected in fractions from Manduca nervous tissues in this study.*

Candidate peptides for the cardioinhibitory factor detected in the *Schistocerca* head and brain tissues could be locustamyoinhibiting peptide, locustamyoinhibin or structurally similar peptides. In *Locusta migratoria*, immunoreactivity to locustamyoinhibin has been detected in the brain and associated structures but not in the ganglia or neurohaemal organs of the nerve cord (Schoofs *et al.* 1994). This is a similar pattern of distribution as the cardioinhibitory factor found in this study. Further work both to test the activity of the purified peptides on the larval heart and to determine the elution time from the HPLC system used for this study, would be required for proof of the identity of the peptides.

Many of the neuropeptides isolated from *Locusta migratoria* have been shown to be myostimulatory in the oviduct of this insect including; Lom-TKs, locustamyotropins I-IV (Lom-MTs I-IV), locustapyrokinins I-II (LPKs I-II), CCAP, proc-

tolin, Lom-AG-MT I-II and some members of the FLRFamide family (Schoofs *et al.* 1993d). A limited survey of the activity of some known insect neuropeptides has shown that, by contrast, the *Manduca* oviduct is responsive to only CCAP and Mas-AT (see Chapter III). The *Manduca* oviduct bioassay was used as a novel screening tool of *Manduca* and *Schistocerca* nervous tissue in a search for other peptides myoactive in this tissue. In comparison to the heart bioassays, the oviduct responded to few fractions. From the *Manduca* abdominal nerve cord and head extracts, the oviduct responded to factors from the fraction in which CCAP would be expected to elute, but also to the previous fraction. In the brain extracts, a peak of activity was seen that eluted close to the putative CCAP myoactivity found in the heads and nerve cords, but that was not identical to it. This may indicate the presence in the brain of component peptides of the CAP2 complex, with other members of this peptide set also stimulating the oviduct or the presence of a different myoactive peptide. In the head and nerve cord extracts the oviduct was also stimulated by fractions corresponding to the expected elution time of Mas-allatotropin but this peak was not seen in the brain extracts. Veenstra and Hagedorn (1993) using a competitive enzyme-linked immunosorbent assay (ELISA) demonstrated that allatotropin is present in the brain, retrocerebral complex and abdominal nerve cord of *Manduca.* The absence of allatotropin activity in the brain extracts as compared to the heads may be due to loss of some retrocerebral complexes during dissection and a subsequent reduction in the amount of allatotropin in the extract.

A factor with strong myotropic activity on the oviduct was detected in a single early fraction from the brain extracts of *Manduca.* This fraction was also strongly cardioactive in the larval heart and active but less so in the adult heart. None of the peptides tested in this study match the bioassay activity and/or the elution time of this peak and therefore the identity of this peptide is unknown.

A number of oviduct myoactive factors were also separated from the *Schistocerca* nervous tissues. None of the active fractions could be identified by reference to the elution time of the synthetic peptides. From the list of *Locusta migratoria* peptides which have been shown to be myoactive in the locust oviduct, Lom-TK II, proctolin and schistoFLRFamide are inactive in the *Manduca* oviduct. In addition Lem-PK and Hez-PBAN, which have some sequence similarity to the locustamyotropins I-IV and the locustapyrokinins I-II are also inactive suggesting that members of these families of peptides are not important for regulation of oviduct motility in *Manduca.* From the known peptides isolated from *Locusta migratoria* the only likely candidates for the *Manduca* oviduct stimulating peptides noted in this study from *Schistocerca gregaria* are CCAP and Lom-AG-MT I and II. CCAP immunoreactive neurones are present throughout the central nervous system of *Locusta migratoria* (Dircksen *et al.* 1991) therefore CCAP oviduct myoactivity could be expected in the brains, heads and nerve cords of *Schistocerca gregaria.* The results from this study however fail to show the presence of oviduct stimulating factors in the fraction in which CCAP would be expected to elute. An explanation could be that CCAP is present in quantities too low for the oviduct to detect. This is supported by the fact that the heart bioassays, which are more sensitive to CCAP, detected cardioactivity in these fractions.

Lom-AG-MT I has close sequence similarity with Mas-AT and myostimulatory activity of this peptide in the *Manduca* oviduct would therefore not be surprising. Neurones containing the Lom-AG-MTs have been immunologically identified in the protocerebrum, deuterocerebrum, optic lobes, frontal ganglion, thoracic and abdominal ganglia of *Locusta migratoria* (Paeman 1991) making detection of these peptides likely in the *Schistocerca* brain, head and nerve cord extracts used in this study. The *Manduca* oviduct bioassay could prove to be a useful tool in the further investigations of these *Schistocerca gregaria* peptides because of the

specificity of the response.

Selected fractions from the first separation of the brain extracts of *Schistocerca gregaria* were subjected to further HPLC analysis. Of particular interest was a factor detected in fractions 25 – 26 in the first separation because of its strong myotropic activity. Following separation 2, these fractions were found to contain a major factor myoactive in all three *Manduca* bioassays and a minor factor active only in the heart bioassays. The major factor elutes in the same fractions as synthetic CCAP under the separation 2 protocol. However it is unlikely to be CCAP as the first separation fraction from which it was isolated did not elute at the same time as CCAP. [His^7]-Corazonin has been isolated from *Schistocerca gregaria* (Veenstra 1991) and in this study corazonin eluted in the same first separation fractions as the major myoactive factor, but corazonin is not myoactive in the oviduct bioassay (Chapter III). The identity of both active fractions cannot be deduced from this study.

The synthetic peptides CCAP and Lom-TK II eluted in fractions 27 – 29 from the first separation. The fractions 27 – 28 were separated with separation protocol 2 and a number of myoactive fractions were seen. The second separation did not successfully resolve the two synthetic peptides but a factor myotropic in all three bioassays eluted in fractions 28 – 29 which was likely to be CCAP. Fraction 30 was cardiosuppressive in the larval heart and myoactive in the adult heart and oviduct bioassays. Further analysis of all the fractions cardiosuppressive in the larval heart by the separation 3 protocol revealed that CCAP was probably present because cardioactivity was detected in the same fraction as CCAP would elute in under the same conditions. The larval cardiosuppressive and the adult cardioacceleratory factors could be separated and are likely to be distinct peptides. Slight evidence of Lom-TK like peptides was seen with fractions 22 and

23 of the third separation being active in the larval but not the adult hearts and synthetic Lom-TK II eluted in fraction 22.

The experiments described here form a preliminary screening of nervous tissues from two insects with three well characterised bioassays from *Manduca sexta.* Although the results are of great interest because of the range of myoactive peptides detected, it was not possible further identify any of the active fractions by purification and sequencing because of time constraints. The results suggest that further investigation with larger amounts of nervous tissue and more HPLC separation steps with a number of different column and solvent conditions, would yield valuable information regarding the peptide regulation of the *Manduca* heart and oviduct and would be likely to result in the sequencing of novel peptides from either or both of the two insect species.

Appendix

5.4.1 Larval *Manduca sexta* Saline.

KCl, 40 mmol l^{-1}, NaCl, 4 mmol l^{-1}, $MgCl_2$, 18 mmol l^{-1}, $CaCl_2$, 3 mmol l^{-1}, Na_2HPO_4, 1.5 mmol l^{-1}, NaH_2PO_4, 1.5 mmol l^{-1}, sucrose 193 mmol l^{-1}.

5.4.2 Adult *Manduca sexta* saline.

As above except sucrose 243 mmol l^{-1}.

5.4.3 Ephrussi and Beadle Saline.

Na^+, 128 mmol l^{-1}, K^+, 5 mmol l^{-1}, Ca^{2+}, 2 mmol l^{-1}, Cl^-, 135 mmol l^{-1}.

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